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ABBREVIATIONS OF TITLES

Abbreviation	*Name of Periodical*
Amer. J. Hyg.	American Journal of Hygiene.
Amer. J. Physiol.	American Journal of Physiology.
Annalen	Justus Liebig's Annalen der Chemie.
Ann. Appl. Biol.	Annals of Applied Biology.
Ann. Chim. Applic.	Annali di Chimica Applicata.
Ann. Physik	Annalen der Physik.
Arch. ges. Physiol.	Archiv der gesammte Physiologie.
Arch. Inter. Physiol.	Archives internationales de Physiologie.
Arch. Mik. Anat. Entwick-Mech. ...	Archiv für mikroscopische Anatomie.
Arch. Protistenk.	Archiv der Protistenkunde.
Astrophys. J.	Astrophysical Journal.
Aust. Journ. Expl. Biol. Med. Sci. ...	Australian Journal of Experimental Biology and Medicine.
Ber.	Berichte der deutschen chemischen Gesellschaft.
Biochem. J.	Biochemical Journal.
Biochem. Z.	Biochemische Zeitschrift.
Boll. chim. farm.	Bolletino Chimico farmaceutico.
Bot. Gaz.	Botanical Gazette.
Bull. Soc. chim.	Bulletin de la Société chimique de France.
Bull. Soc. chim. biol.	Bulletin de la Société de Chimie biologique.
Bull. Soc. mycol.	Bulletin de la Société mycologique.
Chem. Ztg.	Chemiker-Zeitung.
Compt. rend.	Comptes rendus hebdomadaires des Scéances de l'Académie des Sciences.
Compt. rend. Soc. Biol.	Comptes rendus hebdomadaires des Scéances de la Société de Biologie.
Gazzetta	Gazzetta chimica italiana.
Hawaii Agric. Eptl. Sta.	Hawaii Agricultural Experiment Station Bulletins.
Ind. Eng. Chem.	Industrial and Engineering Chemistry.
J. Agric. Research	Journal of Agricultural Research.
J. Agric. Sci.	Journal of Agricultural Science.
Jahrb. Ver. Angew. Bot.	Jahresbericht der Vereinigung für angewandte Botanik.
Jahresber.	Jahresbericht.
J. Amer. Chem. Soc.	Journal of the American Chemical Society.

J. Amer. Soc. Agron.	Journal of the American Society of Agronomy.
J. Bact.	Journal of Pathology and Bacteriology
J. Biol. Chem.	Journal of Biological Chemistry.
J. C. S.	Journal of the Chemical Society.
J. Gen. Physiol.	Journal of General Physiology.
J. Ind. Chem. Soc.	Quarterly Journal of the Indian Chemical Society.
J. Ind. Eng. Chem.	Journal of Industrial and Engineering Chemistry.
J. Ind. Inst. Sci.	Journal of the Indian Institute of Science.
J. Inst. Elec. Eng.	Journal of the Institution of Electrical Engineers.
J. Inst. Eng., India	Journal of the Institution of Electrical Engineers, India.
J. Land.	Journal für Landwirtschaft.
J. Med. Res., N. S.	Journal of Medical Research, New Series.
J. Pharm. Chim.	Journal de Pharmacie et de Chimie.
J. Physical Chem.	Journal of Physical Chemistry.
J. Physiol.	Journal of Physiology.
J. Physiol. Chem.	Journal of Physiological Chemistry.
J. pr. Chem.	Journal für praktische Chemie.
J. Sci. Ass. Vizianagram	Journal of the Vizianagram Science Association.
J. Soc. Chem. Ind.	Journal of the Society of Chemical Industry.
Kolloid Z.	Kolloid-Zeitschrift.
Monatsh.	Monatshefte für Chemie und verwandte Teile ander Wissenschaften.
Naturwiss.	Die Naturwissenschaften.
N. Cimento	Il Nuovo Cimento.
N. Y. Agr. Exp. Sta. Bull.	New York Agricultural Experimental Station Bulletins.
Osterr. botan. Z.	Oesterreichische botanische Zeitung.
Pharm. Indica	Pharmacologica Indica.
Phil. Mag.	Philosophical Magazine.
Phys. Rev.	Physical Review.
Proc. Inst. Mech. Eng.	Proceedings of the Institution of Mechanical Engineers.
Proc. Phys. Soc.	Proceedings of the Physical Society of London.
Proc. Roy. Soc.	Proceedings of the Royal Society.
Q. J. M. S.	Quarterly Journal of Microscopical Science.

Rec. trav. chim.	Recueil des travaux chimiques des Pays-Bas.
Russ. Pharm. Ztg.	Russische pharmazeutische Zeitung.
Sci. Rep. Agri. Res. Inst., Pusa	Scientific Reports of the Agricultural Research Institute, Pusa.
Soil Sci.	Soil Science.
Trans. Faraday Soc.	Transactions of the Faraday Society.
Univ. Calf. Pub. Zool.	University of California Publications in Zoology.
Woch. Brau.	Wochenschrift für Brauerei.
Z. anorg. Chem.	Zeitschrift für anorganische und allgemeine Chemie.
Z. Elektrochem.	Zeitschrift für Elektrochemie.
Zent. Bakt.	*Bakteriologisches Zentralblatt.*
Z. physikal. Chem.	Zeitschrift für physikalische Chemie, Stöchiometrie und Verwandtschaftslehre.
Z. physiol. Chem.	Hoppe-Seyler's Zeitschrift für physiologische Chemie.
Z. Ver. deut. Zucker-Ind.	Zeitschrift des Vereins der deutschen Zucker-Industrie.

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PHOTOTROPIC COMPOUNDS OF MERCURY.*

By Bh. S. V. Raghava Rao and H. E. Watson

It has previously been shown by one of us (*Nature*, 1923, **111**, 775) that the yellow compound HS.Hg.CNS prepared by the action of thio-urea and ammonium sulphocyanide upon a concentrated solution of mercuric chloride in acetic acid in presence of an oxidising agent is very rapidly turned black by exposure to light and regains its original colour after standing for a short time in the dark or on heating. The compounds Cl.Hg.CNS, Br.Hg.CNS, and I.Hg.CNS, the first of which was originally prepared by McMurthy (*J. C. S.*, 1889, **55**, 50) have also been shown to possess similar properties.

The corresponding cyanates and selenocyanides have now been prepared, as well as a number of double sulphides, and the phototropic properties of the more sensitive compounds have been examined in a quantitative manner. Attempts to prepare similar tellurium compounds have not been successful.

EXPERIMENTAL.

These compounds are all insoluble or very slightly soluble in water and accurate analytical results were somewhat difficult to obtain. The figures given are sufficient to identify the compounds with the suggested formulae but are not accurate enough to indicate the presence of small quantities of impurities. As it was considered possible that the phototropic properties might be considerably modified by traces of foreign matter a comparison was made in several cases between preparations of the same compound made at different times and by alternative methods, but the differences observed were always small and hardly beyond the limit of experimental error.

In conducting the analysis, mercury was estimated as sulphide by Volhard's method after solution of the compound in aqua regia. Halogens were determined by decomposing the compounds with a large excess of fusion mixture, sulphur by heating with sodium carbonate and potassium nitrate and estimation as barium sulphate, and selenium by solution in aqua regia followed by evaporation with hydrogen chloride and precipitation with sulphur dioxide. When selenium was present, mercury was estimated as mercurous chloride.

* Reprinted from the *Journal of Physical Chemistry*, 1928, **32**, 1354.

CYANATES.

The compounds HS.Hg.CNO and HO.Hg.CNO could not be obtained by treating a mercury salt with ammonium cyanate and thio-urea or urea. The halides Cl.Hg.CNO and Br.Hg.CNO were prepared by boiling together equimolecular proportions of mercuric cyanate and the mercury halide for some time and filtering. The complex salts crystallised out. The iodine compound could not be produced in this way but was obtained as an insoluble residue when mercuric cyanate was boiled with a slight excess of potassium iodide, the yield being quantitative.

The bromine and iodine compounds were also formed by the action of an alcoholic solution of the halogen upon mercurous cyanate.

Chloro-mercuric cyanate is a pale brown substance. It turns dark brown in about half an hour in diffused daylight and regains its original colour after keeping it in the dark for two days. Analysis gave Hg, 72·3; Cl, 12·7: Cl.Hg. CNO requires Hg, 72·0; Cl, 12·8.

Bromo-mercuric cyanate is yellow and darkens more rapidly than the chloro-compound. The time for reversal is about the same. Analysis gave Hg, 62·9; Br, 25·1: Br.Hg.CNO requires Hg, 62·1; Br, 24·8.

Iodo-mercuric cyanate has a bright golden yellow colour and blackens completely in about 3 minutes in diffused daylight. The reversal takes place slightly faster than in the two preceding cases. Analysis gave Hg, 53·6; I, 34·2 : I.Hg.CNO requires Hg, 54·2; I, 34·4.

To avoid loss of iodine during analysis, decomposition of this compound was effected by gentle heating with potassium hydroxide.

SELENOCYANIDES.

The compounds HS.Hg.CNSe and HSe.Hg.CNSe as also the halogen double salts were obtained without difficulty. The compound HSe.Hg.CNSe is not phototropic.

Chloro-mercuric selenocyanide has been perepared by Rosenheim and Pritz (*Z. anorg. Chem.*, 1909, **63**, 275) by adding potassium selenocyanide to a concentrated solution of mercuric chloride. It can also be prepared by treating mercuric selenocyanide with a dilute solution of potassium chloride. It is very feebly phototropic. Analysis gave Hg, 59·2; Se, 23·8; Cl, 10·1: Cl.Hg.CNSe requires Hg, 58·7; Se, 23·3; Cl, 10·4.

For analysis the compound was decomposed with potassium hydroxide.

Bromo-mercuric selenocyanide was prepared by treating mercuric selenocyanide with a dilute solution of potassium bromide. It is a very finely divided yellow powder and blackens in about 2 minutes in diffused daylight. The original colour returns in about 24 hours at room temperature (30°). Analysis gave Hg, 51·5; Se, 20·3; Br, 20·8: Br.Hg.CNSe requires Hg, 51·9; Se, 20·6; Br, 20·8.

Iodo-mercuric selenocyanide prepared in an analogous manner is an orange powder. It darkens in less than 2 minutes. The reversal is much slower than with the bromine compound. Analysis gave Hg, 45·7; Se, 18·2; I, 29·1; I.Hg.CNSe requires Hg, 46·2; Se, 18·3; I, 29·3.

Hydrosulpho-mercuric selenocyanide is formed as a light brown substance when a slow current of hydrogen sulphide is passed through mercuric selenocyanide suspended in water. It is very feebly phototropic taking an hour and a half to darken in diffused daylight. It returns to its original colour in the dark in about 3 days. Analysis gave Hg, 60·2; Se, 23·9; S, 9·5: HS.Hg.CNSe requires Hg, 59·2; Se, 23·4; S, 9·4.

Hydroseleno-mercuric selenocyanide is a brown compound which appears to be HSe.Hg.CNSe from its method of formation (i.e. by passing H_2Se into Hg $(CNSe)_2$ suspended in water) and is not phototropic.

Hydroseleno-mercuric sulphocyanide similarly formed as a grey powder on passing H_2Se into $Hg(CNS)_2$ suspended in water is also not phototropic.

DOUBLE SULPHIDES OF MERCURY.

The two compounds $Hg_3S_2Br_2$ and $Hg_3S_2I_2$ first prepared by Franceschi (*Boll. chim. farm.*, 1916, **55**, 481) have been found to be phototropic by Varahalu (*J. Sci. Ass. Vizianagram*, 1924, **1**, 101). The corresponding chlorosulphocyano- and selenocyano-compounds have been prepared. An attempt to prepare the corresponding cyanide and cyanate ended in failure.

Trimercuric-disulpho-dichloride has been obtained as a white crystalline substance by adding potassium chloride solution to a solution of freshly precipitated mercuric sulphide in mercuric acetate. It has also been obtained by adding hydrogen sulphide solution to mercuric chloride solution acidified with hydrogen chloride (Rose and Palm, *Russ. Pharm. Ztg.*, **1862**, 120). It turns black in about 10 minutes in diffused daylight. Reversal takes two days at room temperature.

Trimercuric-disulpho-disulphocyanide is prepared from mercuric sulphide dissolved in mercuric acetate by precipitation with potassium sulphocyanide solution. It is yellow at first but becomes slightly greenish. It darkens in diffused daylight in about 2 to 3 minutes and regains its original colour in the dark after two days. Analysis gave Hg, 76·6; S, 16·5: $Hg_3S_2(CNS)_2$ requires Hg, 77·0; S, 16·4.

Trimercuric-disulpho-diselenocyanide is prepared in an analogous manner by using potassium selenocyanide solution for precipitation. It is a brown solid which turns crimson on exposure to light for about six minutes. It regains its original colour in about 40 hours in the dark.

For determination of sulphur the substance was fused with a mixture of potassium nitrate and sodium carbonate; the fusion extract was repeatedly boiled with concentrated hydrogen chloride in which selenium was precipitated and filtered. From the filtrate the sulphate was precipitated in the usual way. Analysis gave Hg, 68·4; Se, 17·9; S, 7·2: $Hg_3S_2(CNSe)_2$ requires Hg, 68·7; Se, 18·1; S, 7·3.

DOUBLE SELENIDES AND OXIDES.

The selenides corresponding with the halogen double sulphides have also been prepared and examined together with one oxygen compound $Hg_3O_2(CNS)_2$.

Trimercuric-diseleno-dichloride is formed as a white precipitate when a slow stream of hydrogen selenide is passed into an aqueous solution of mercuric chloride (Uelsmann, *Jahresber.*, **1860**, 92). It is feebly phototropic, taking nearly two hours to darken in diffused daylight. Reversal takes about 2 days at room temperature.

Trimercuric-diseleno-dibromide is obtained as a pale yellow substance by passing hydrogen selenide into a solution of mercuric bromide. It takes 40 to 50 minutes to darken completely in diffused daylight, while in the direct sun the change is brought about in 8 to 10 minutes. The reverse change takes about 36 hours. Analysis gave Hg, 65·4; Br, 17·6; Se, 17·2: $Hg_3Se_2Br_2$ requires Hg, 65·4; Br. 17·4; Se, 17·2.

Trimercuric-diseleno-diiodide is obtained as a light brown substance by treating either trimercuric-diseleno-dichloride or the dibromide with excess of potassium iodide solution. It darkens in less than 5 minutes in diffused daylight and takes the same time as the dibromide to recover. Analysis gave Hg, 59·6; I, 25·3; Se, 15·8: $Hg_3Se_2I_2$ requires Hg, 59·3; I, 25·0; Se, 15·6.

Trimercuric-dioxy-disulphocyanide is formed as a yellow powder when mercuric sulphocyanide is treated with ammonia. It is very

feebly phototropic taking 3 to 4 hours to darken in sunlight. The recovery, however, is complete in three days. Analysis gave Hg, 80·5; S, 8·7: $Hg_3O_2(CNS)_2$ requires Hg, 80·3; S, 8·5. No water was eliminated on decomposing the compound by heat.

Table I gives a list of these compounds and their properties.

TABLE I.

Phototropic Mercury Compounds.

Substance	Colour	Darkening time, minutes	Time for recovery, days
Cl.Hg.CNO ...	Pale brown ...	30	2
Br.Hg.CNO ...	Pale yellow ...	15	2
I.Hg.CNO ...	Golden yellow ...	3	1·5
Cl.Hg.CNS ...	White ...	20	2
Br.Hg.CNS ...	White ...	2	2
I.Hg.CNS ...	Orange yellow ...	1	1·5
Cl.Hg.CNSe ...	Pale yellow ...	20	1·5
Br.Hg.CNSe ...	Yellow ...	2	1
I.Hg.CNSe ...	Orange ...	<2	1·5
HS.Hg.CNS ...	Lemon yellow ...	<1	2
HSe.Hg.CNS ? ...	Grey ...	not ph.	...
HS.Hg.CNSe ...	Pale brown ...	90	3
HSe.Hg.CNSe ? ...	Brown ...	not ph.	...
$Hg.Cl_2$.,2HgS ...	White ...	10	2
$Hg.Br_2$.,2HgS ...	Pale yellow ...	3	2
$Hg.I_2$.,2HgS ...	Orange yellow ...	<1	2
$Hg.Cl_2$.,2HgSe ...	White ...	120	2
$Hg.Br_2$.,2HgSe ...	Pale yellow ...	45	1·5
$Hg.I_2$.,2HgSe ...	Pale brown ...	5	1·5
$Hg(CNS)_2$.,2HgO ...	Yellow ...	200	3
$Hg(CNS)_2$.,2HgS ...	Yellow green ...	3	2
$Hg(CNSe)_2$.,2HgS ...	Brown ...	6	1·5

It is interesting to note that although the darkening times for these compounds vary through a very wide range, the times of recovery are all of the same order.

SPECTROSCOPIC EXAMINATION.

The more active of these compounds have been exposed to light in a spectroscope in order to compare the effect of the different wave lengths. Similar experiments have been made by H. Stobbe (*Annalen*, 1908, **359**, 1) with the fulgides using a special spectroscope which gave very intense illumination. In the present case owing to the greater sensitivity of the compounds examined an ordinary spectroscope could be used.

The substance was mixed with sufficient 0·1 per cent. solution of gelatine to enable it to flow freely, coated on a glass plate and dried in the dark. Strips of the plate were then exposed for varying times in the spectroscope to the light from a 100 c.p. pointolite lamp. For examination in the ultra-violet a quartz spectroscope was used with an iron arc as source of light, but this was somewhat difficult to keep constant in intensity and consequently most of the measurements were made in the visible region.

As the compounds under investigation regained their original colour in the dark, it was anticipated that exposure of the darkened substance to red light would have a bleaching effect and this was found to be the case, the general action of light being bleaching (of the substance after exposure to white light) in the red or infra-red, a neutral zone in the yellow or orange and darkening in the green and blue.

In order to preserve a permanent record of the appearance of the plates after exposure, they were illuminated with light of a wave-length which had no action upon them by means of filters and photographed upon a panchromatic plate.

Fig. 1A shows the curve obtained for the compound HS.Hg.CNS when the reciprocals of the times of exposure are plotted against the limits to which visible darkening extended after exposure. The portion of the curve below the zero line indicates that the previously exposed plate was bleached in the time noted. It will be seen that the curve has a flat top and very steep sides so that the chief sensitive region is distributed over a comparatively wide band of frequency but the limits are fairly sharply defined. This could be distinctly seen on the plates to which longer exposures had been given in which two dark zones were visible, the central darker one corresponding with the above

The darkening in the ultra-violet was apparently uniform and extended to the limit of transmission of the spectroscope. Similarly the bleaching effect had no evident maximum, and examination with a grating (for the loan of which we are greatly indebted to Dr. E. P. Metcalfe of the Central College, Bangalore) into the infra-red did not disclose the existence of a non-sensitive region.

Fig. 1 also shows sensitivity curves for seven other substances. They are all of a similar nature except the one for the compound $Hg_3S_2Cl_2$ which exhibits fairly uniform darkening in the blue and ultra-violet regions with a sharp cut-off in the green, but no maximum. The exact shape shown for the curves on the blue side may be in some cases not quite correct owing to the absorption of the glass, although checked as accurately as possible with the quartz spectrograph. The limits of sensitiveness at the red end are however well defined and it will be noticed that the substitution of Cl by Br and Br by I in the

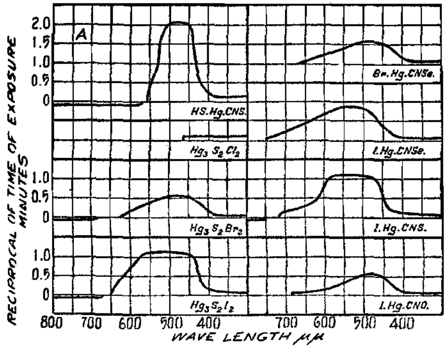

FIG. 1

series $Hg_3S_2X_2$ results in a shift of the maximum and of the excitation limit towards the red. A similar shift takes place on substitution of I for Br in the compound Br.Hg.CNSe, and also in the series I.Hg.CNO, I.Hg.CNS, I.Hg.CNSe.

The points at which reversal begins are also marked when they could be ascertained, but in several cases they were too far in the red to be detected. Some observations were also made with the compounds $Hg_3S_2(CNS)_2$ and $Hg_3S_2(CNSe)_2$. The region of maximum sensitiveness for the former extended from 4550–5000 A.U. thus coinciding nearly with that for the compound $Hg_3S_2Br_2$ but the

sensitive region reached only to 5700 and reversal started at 6000 as compared with 6250 and 6850 for the bromine compound. The selenium compound had a wider maximum sensitiveness (4800–5575) further in the red than that of the sulphur compound and the sensitive region reached to 6350. Reversal could not be detected in the spectroscope.

Action of the Medium.

It has been mentioned that gelatine was used to attach the varying compounds to the glass plates. Preliminary experiments were made to determine the effect of this substance on the sensitiveness using the compound HS.Hg.CNS. Plates were coated with suspensions of the compound in gelatine solutions of different concentrations and exposed in the spectroscope for varying periods. About 1 gram of substance was mixed with 10 c.c. of gelatine solution so that in the case of a one per cent. solution the weight of gelatine amounted to 10 per cent. of the weight of the compound. Table II shows the region in which perceptible darkening occurred.

TABLE II.

Effect of Concentration of Gelatine upon Sensitiveness of

HS.Hg.CNS.

Conc. of Gelatine	Region darkened (A.U.) in 2 minutes	4 minutes	8 minutes
0·23	4520–5340	4270–5570	4100–5630
0·47	4470–5340	4230–5570	4100–5630
0·93	4410–5410	4210–5640	4040–5640
1·86	4470–5340	4270–5580	4070–5630

The quantity of gelatine has thus a distinct influence upon the sensitiveness, there being a maximum at a concentration of about 10 per cent. of the weight of the substance.

It has been observed by M. L. Dey (*Nature*, 1923, **112**, 240) that a gelatine emulsion of $Hg_3S_2I_2$ blackens more rapidly than the pure substance but he also states that the reverse change is completely inhibited. As this latter observation was not confirmed by our experiments it was decided to examine the phenomenon in somewhat greater detail.

Plates were coated with three different compounds with the addition of small quantities of binding agents and each set exposed to diffused daylight until the coloration reached a standard shade. The times taken to attain this colour were noted. The plates were then put in an electrically heated oven at either 50° or 70° for 45 or 35 minutes respectively and the extent to which bleaching had taken place at the end of the time was noted. The relative order of recovery could thus be judged. Table III shows the results of these experiments.

In the experiments marked (*a*) reversal was effected at 50° and in those marked (*b*) at 70°. As the order of reversal was the same at both temperatures in every case, only one set of figures has been given. There is no relation between the times (*a*) and (*b*) or for the figures which refer to different compounds. The only results which are comparable are those for the same substance with different media shown in one vertical column. From these it may be seen that the velocity of coloration depended in every case upon the medium only and that while agar agar, gelatine and collodion in the amounts employed exerted a marked accelerating influence, gum arabic produced a retardation. All the media had a retarding influence upon the reverse reaction.

TABLE III.

Action of Different Media upon Sensitivity.

Medium	HS.Hg CNS			I.Hg.CNS			$Hg_3S_2I_2$		
	Secs. required for coloration		Order of reversal	Secs. required for coloration		Order of reversal	Secs. required for coloration		Order of reversal
	a	*b*		*a*	*b*		*a*	*b*	
Agar Agar 0·1 per cent.	90	150	3	280	540	3	250	240	3
Gelatine 0·1 per cent.	120	200	5	300	600	3	270	260	5
Collodion 0·06 per cent.	155	255	3	330	660	2	280	270	3
Nil	180	300	1	360	720	1	300	285	1
Gum arabic 0·13 per cent.	230	420	2	390	810	3	340	330	2

Sensitisation by Means of Dyes.

As all the substances examined showed marked differences of sensitivity to light of different wavelengths it was considered probable that the position of the sensitive region would be altered by the action of dyes as it is in the case of the photographic plate.

In order to test this a series of experiments was made with the compound HS.Hg.CNS. Plates coated with this substance with a small quantity of gelatine as adhesive were dipped in dilute solutions (about 1 in 20,000) of various dyes for 1 minute and then dried. Experiments with erythrosin in which dye solutions of very different concentrations were used, indicated that the effect of concentration upon the sensitiveness was not appreciable provided that more than a certain minimum amount of dyestuff was used. The plates were exposed to the light from a pointolite lamp in the spectroscope as in the former experiments, both without previous exposure to light and after slight fogging in daylight.

In all cases the dyes had a distinct effect. The region in which darkening occurred was extended towards the red while the zone of reversal was also affected. The nature of these changes is shown in Fig. 2, the portion of the curve above the line indicating darkening and the portion below, bleaching. It is interesting to note that the two regions overlap. In the blue region it is possible even in the less sensitive parts to obtain practically complete blackening with sufficient exposure. Where the two curves overlap blackening is not so complete and only a well-darkened plate shows the bleaching effect. It is evident that in this case the velocities of the direct and reverse reactions are comparable so that an equilibrium is established.

An examination of the curves shows that the blue and green dyes sensitise furthest towards the red but that the sensitising effect of eosin and erythrosin is more marked.

An interesting phenomenon was noticed when these dyed plates were exposed to light of different wavelengths. The colour produced by light in the normal region of maximum sensitiveness (blue-green) differed distinctly in shade from the colour in the orange region which resulted from the presence of the dye. Without dye, there would have been a bleaching effect in this region. The difference was noticeable with all the dyes used but was most conspicuous in the case of eosin and erythrosin. The effect may be due to a difference in the state of molecular aggregation but no definite information on the subject is yet available.

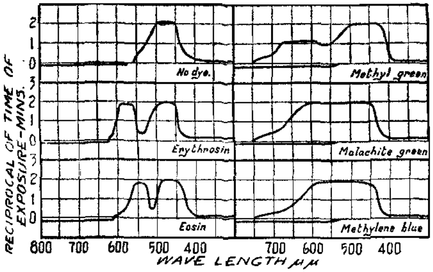

Fig. 2.

A kindred effect was observed when a dyed plate previously exposed in the spectrograph was exposed to daylight. The unexposed portion darkened more rapidly than the part which had been exposed to red light so that after a short interval the former was actually darker than the latter. This appears to indicate that preliminary exposure to red light desensitises the material. The effect was similar when the second exposure was made through a blue screen.

Temperature Coefficients of Direct and Reverse Reaction Velocities.

A number of measurements have been made with the object of determining as far as possible the temperature coefficients of the reactions in the light and in the dark. Two compounds HS.Hg.CNS and I.Hg.CNS were studied and the method adopted was to measure the time required to change from one tint to another under varying conditions. As it was not possible to use darkened specimens of the substances themselves for comparison purposes three standard mixtures of calcium chromate, red lead and lamp black were made which closely matched the tints of both compounds at three stages of darkening. Plates coated with these mixtures were mounted side by side with similar plates coated with the substance under observation, no medium being used, and placed inside a small electrically heated oven with a glass front. For the direct reaction the plates were illuminated with a 500 c.p. pointolite lamp, and for reversal the oven was kept in the dark and observations made from time to time through a red glass.

The accuracy with which the time of exact matching of the tints could be judged was not very great and consequently a number of readings were taken in each case. The mean values for the times of reversal are shown in Table IV.

TABLE IV.

Times for Reversal at Different Temperatures.

Substance	Tints	Time required mins.			t_{40}/t_{50}	t_{50}/t_{60}
		40°	50°	60°		
HS.Hg.CNS ...	3 to 2	61	35	18	1·75	1·95
	2 to 1	70	39	21	1·8	1·85
I.Hg.CNS ...	3 to 2	42	22	12	1·9	1·85
	2 to 1	48	29	13	1·9	1·9

As the change in tint from one standard to another corresponds with the transition of a definite proportion of the substance from the dark to the light variety, the times taken to change from one colour to another when the shades do not differ very widely may be taken as approximately inversely proportional to the reaction velocity. Hence the figures in the last two columns represent the temperature coefficients for a change of 10°. It will be observed that they approximate to 1·9 which is a usual figure for a chemical reaction of the ordinary type.

The reaction in light is complicated by the fact that at the higher temperatures the rate of the reverse action is of the same order. It is shown in the next section that the time of darkening is inversely proportional to the intensity of the incident light and hence it is probable that the amount of darkening is proportional to the time of exposure for a limited range. The same does not necessarily hold for the reaction in the dark but it may be assumed to be approximately correct for a small change of colour. Hence if t_1 is the time for a given colour change in the light and t_2 in the dark, the amounts changed in unit time are proportional to $1/t_1$ and $1/t_2$ and when both reactions take place simultaneously the change is proportional to $1/t_1 - 1/t_2$; so that if t is the observed time, $1/t = 1/t_1 - 1/t_2$ giving t_1 in terms of t and t_2. t_2 is known from the results in Table IV so that t_1 can be calculated. Table V gives the results of the observations.

TABLE V.

Times for Darkening at Different Temperatures.

Substance	Tints	t obs. mins.			t_1 mins.		
		40°	50°	60°	40°	50°	60°
HS.Hg.CNS ...	1 to 2	5·3	5·6	6·2	4·9	4·9	4·8
	2 to 3	5·1	5·4	6·0	4·7	4·7	4·5
I.Hg.CNS ...	1 to 2	10·1	11·3	13·0	8·3	7·8	6·5
	2 to 3	9·7	10·9	12·1	7·8	7·3	6·0

From these it appears that the velocity of transformation of the compound HS.Hg.CNS is unaffected by temperature while for I.Hg.CNS the temperature coefficient is of the order 1·05 from 40° to 50° and 1·2 from 50° to 60°. In the later case the correction for the reverse reaction is very large, particularly at the higher temperature and it is probable that the simple formula used to obtain t_1 is incorrect. It seems likely therefore that the temperature coefficient for both the substances is in the neighbourhood of 1, the characteristic value for a purely photochemical reaction. These results correspond with those of Padoa and Tabellini (*Gazzetta*, 1915, **451**, 10) who, in studying the phototropic transformation of piperil-*o*-tolylosazone have shown that the temperature coefficient of the direct photochemical reaction is 1·06 while in the dark the corresponding figure is 2.

THE RELATION BETWEEN LIGHT FLUX AND PHOTOTROPIC CHANGE.

If the quantity of a substance which undergoes phototropic change is proportional only to the total amount of light energy falling upon it, the time of exposure necessary to produce a constant change should be inversely proportional to the light intensity. This has been found to be the case for the substances HS.Hg.CNS and I.Hg.CNS.

TABLE VI.

Relation between Time of Exposure and Light Intensity.

Tint 1			Tint 2			Tint 3		
t mins.	I	I.t	t	I	I.t	t	I	I.t
				HS.Hg.CNS				
2	100	200	6	67	402	8	100	800
4	51	204	8	50	400	10	80	800
6	33·5	210	10	40	400	12	67	804
8	25	200	12	33·5	402	14	57	798
10	20	200	14	29	406	16	50	800
12	16·5	198	16	25	400	20	40	800
14	14·5	203	20	20	400	25	32	800
20	10	200	30	13·5	405	30	27	810
30	7	210	60	7	420	45	18	810
60	3·5	210	...	...	...	60	13·5	810
				I.Hg.CNS				
4	100	400	8	100	800	20	100	2000
6	67·5	405	10	82	820	25	81	2025
8	50	400	12	67·5	810	30	67	2010
10	40	400	16	50	800	35	58	2030
12	34·5	414	20	40	800	40	50	2000
15	26·5	398	30	27	810	45	45	2025
30	13·5	405	60	13·5	810	60	33·5	2010
60	7	420	120	6·5	780	120	17	2040

Measurements were made by coating glass plates with the compounds using a very dilute gelatine solution (0·01 per cent.) as binder. The plates were heated for 2 hours at 60° in the dark in order to reverse small quantities of the substance which might have been darkened by accidental exposure to light during preparation and then exposed

through a calibrated neutral tint wedge to the light from a 500 c.p. pointolite lamp. Exposures of varying duration were given at a constant distance from the source of light and a strip of graduated intensity thus obtained for each exposure. The portion of this strip which just matched a standard tint was noted and the intensity of the transmitted light at that point ascertained from the calibration of the wedge. Table VI shows the times of exposure and the intensity of the light required to produce a coloration matching each of three different standard tints (the same as those used in the experiments upon the temperature coefficients).

The product I.t is in all cases constant and there is no indication of any period of induction.

SUMMARY.

1. Twenty mercury compounds of the types X.Hg.CNY and $Hg.X_2$, 2HgY where X is Cl, Br, I, HS, HSe, CNS or CNSe and Y is O, S or Se have been prepared and found to be phototropic.

2. By spectroscopic examination of 10 of the more sensitive of these compounds it has been found that with one exception they are all darkened by exposure to light of wavelength of the order of 5500 A.U. and less, slight sensitiveness extending into the ultra-violet to the limit of transmission of quartz. A broad but well defined maximum of sensitiveness is found, usually in the green. The position of the maximum for related compounds tends to move towards the red with increase in molecular weight.

3. If the compounds are previously darkened by exposure to light, reversal can be effected by exposing to red light in the spectroscope. No maximum could be detected, but there was a limiting wave length on the short wave side beyond which no reversal occurred. In several cases this point was too far in the red to be determined. Between the reversal region and the region sensitive to light was a neutral zone in which no action took place. Reversal was in all cases brought about by heat.

4. The effect of various media has been examined. In most cases the direct reaction is accelerated by a medium such as gelatin, but the reverse reaction is always retarded.

5. The region sensitive to light can be extended towards the red by dyeing the compounds as in the case of the photographic plate.

6. The temperature coefficients of the direct (light) and reverse (dark) reactions have been measured for two compounds and found to be approximately 1·0 and 1·9 respectively.

7. The time of exposure necessary to produce a given change has been found inversely proportional to the intensity of the light. No induction period has been observed.

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PHOTOELECTRIC EMISSION FROM PHOTOTROPIC MERCURY COMPOUNDS.

By Bh. S. V. Raghava Rao and H. E. Watson.

Anaduzzi and Padoa (*N. Cimento*, 1912, **3**, 41), studying the photoelectric emission from benzaldehydephenylhydrazone and other similar phototropic compounds, concluded that the photoelectric current varies with the change in colour. Gallaghere (*Bull. Soc. chim.*, 1921, iv, **29**, 961) obtained similar results with phototropic arylamines. Both authors measured the photoelectric effect by determining the rate of discharge of an electrometer, and thus were unable to detect any rapid changes which might have occurred in the course of their experiments.

We have recently described a number of mercury compounds which exhibit a high degree of phototropic activity (*J. Physical Chem.*, 1928, **32**, 1354) and it seemed possible that these might also have a large photoelectric emission. The method of measuring photoelectric currents described by Toy, Edgerton and Vick (*Phil. Mag.*, 1927, **3**, 482) in which use is made of a thermionic valve appeared very suitable for the purpose, as it is possible by its means to determine the instantaneous current even when very small. Applying this method we have measured the emission from twelve compounds and have found that in nearly every case those prepared in the dark are not photoelectrically active; but after exposure to light, emission of electrons takes place on irradiation with ultra-violet light. A possible explanation is that the light energy is at first expended in effecting a transformation of one form to the other, and it is only when light falls on a transformed particle that an electron can be liberated. This simple hypothesis is, however, not altogether satisfactory in view of the fact that the maximum phototropic activity is found in the visible region while photoelectric activity is confined to the ultra-violet. For the moment no other explanation is forthcoming.

EXPERIMENTAL.

The arrangement of the apparatus is shown diagramatically in Fig. 1. The photoelectric cell A was supported on blocks of paraffin and connected between the grid and plate of the thermionic triode V. Ordinarily 160 volts were applied to the plate and the steady anode current balanced by means of the potentiometer P so that the galvanometers G_1 G_2 showed no deflection. P consisted of a fixed resistance of 700 ohms and variable 12 and 1 ohm resistances in series with it so

that a very fine adjustment was possible. G_1 was a pointer galvanometer and G_2 a mirror instrument giving a deflection of 1 cm. for 1×10^{-7} amp. These were fitted with suitable shunts and switches and indicated any change in anode current.

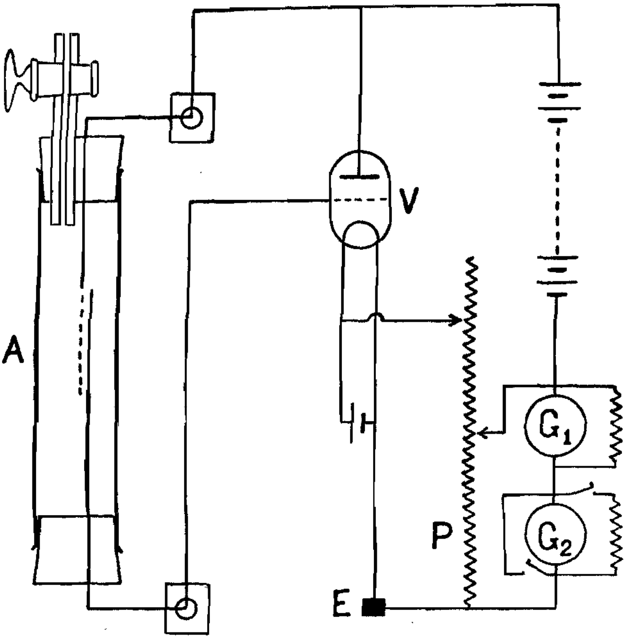

FIG. 1.

The thermionic valve has been used in conjunction with a photoelectric cell by Rosenberg (*Naturwiss.*, 1921, **9**, 359, 389), du Prell (*Ann. Physik*, 1923, **70**, 199) and Ferrie, Jouaust and Mesny (*Compt. rend.*, 1923, **177**, 847). All these authors arranged the cell in such a way that the photoelectric current charged the grid negatively and the system thus behaved as an electroscope of small capacity. Equilibrium was reached when the current from the cell was equal to the leakage, and by improving the insulation it was found possible to

obtain effective magnifications of 10^6 and even more. Du Prell investigated the matter in considerable detail and drew attention to the harmful effect of grid current.

In the experiments of Toy, Edgerton and Vick the photoelectric cell charges the grid positively and thus the conditions are different. These authors offer no explanation for the action of the cell and consequently it was considered desirable to ascertain the exact reason for the constancy of the change in anode current for a given photoelectric current and to ensure that the relation between these two quantities was a linear one.

It seemed likely *a priori* that equilibrium would be obtained when the photoelectric current was equal to the grid current. The usual grid current characteristic A, Fig. 2, approaches the axis asymptotically and does not yield much information. The potential of the free grid

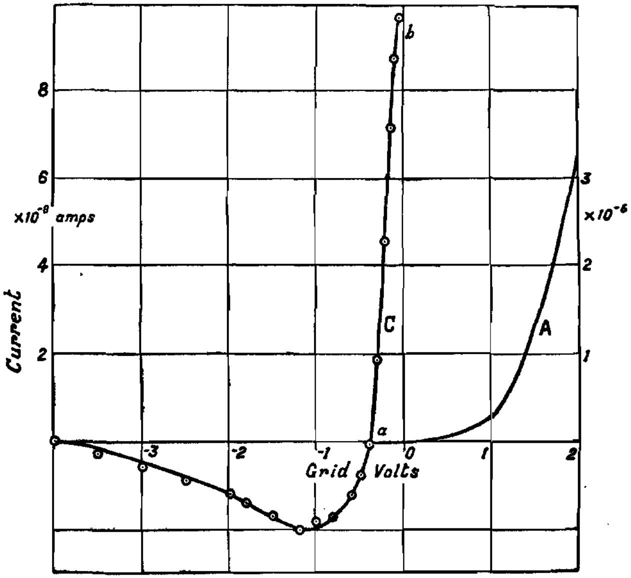

FIG. 2.

was found to be – 0·4 volts and further measurements were made of the very small current in this region by the usual method of biassing the grid through a high resistance (4 megohms), short circuiting the resistance and measuring the change in anode current. The curve obtained is shown in Fig. 2 (*c*), the grid current being reversed in part owing to incomplete evacuation of the valve.

The point *a* where the grid current is zero gives the potential of the 'free' grid, no change in anode current resulting if the grid is disconnected when biassed to this voltage. The slope of the curve from *a* to *b* determines the sensitiveness of the valve. Its value in the neighbourhood of *a* is $1{\cdot}8 \times 10^{-7}$ amp. per volt and as one volt change on the grid at this point results in a change in anode current of $0{\cdot}33 \times 10^{-3}$ amp. the current magnification of the system is about 1,800.

As the curve from *a* to *b* is approximately linear and measurements are made over only a small range (1 mv. grid potential) it would be expected that the change in anode current would be proportional to the photoelectric current. This was tested by exposing a silver cell to the mercury arc at different distances and Fig. 3 shows that the assumption is correct within the limit of experimental error.

The actual valve used to which the curves refer was a Cleartron 25B, impedance 33,000 ohms, amplification factor 11. This was selected as being the most sensitive of a number of valves of different types. The suitability of a valve for this purpose has since been investigated in some detail and will form the subject of a separate communication. In making the above calculations the leakage between grid and filament has been neglected. This was measured and found not to influence the results to any appreciable extent.

The photoelectric cell.—The substance to be examined was made into a thin paste with water and floated over a piece of platinum foil measuring 16 × 9 mm. On drying, the coating adhered to the platinum sufficiently to allow handling. Platinum was used as a support as it would not react with the mercury compounds and also because it has been shown by Woodruff (*Physical Rev.*, 1925, **26**, 655) that the photoelectric emission from a platinum surface is negligible under the conditions of our experiments. A piece of nickel gauze the same size as the platinum was used as anode. In order to secure the highest possible insulation, the wires supporting the electrodes were passed through corks in the opposite ends of a quartz tube 1 cm. in diameter, the joints being made airtight with a beeswax-resin mixture. At one end of the tube a small tap was inserted for evacuation. The assemblage is shown in Fig. 1. With this arrangement it was possible

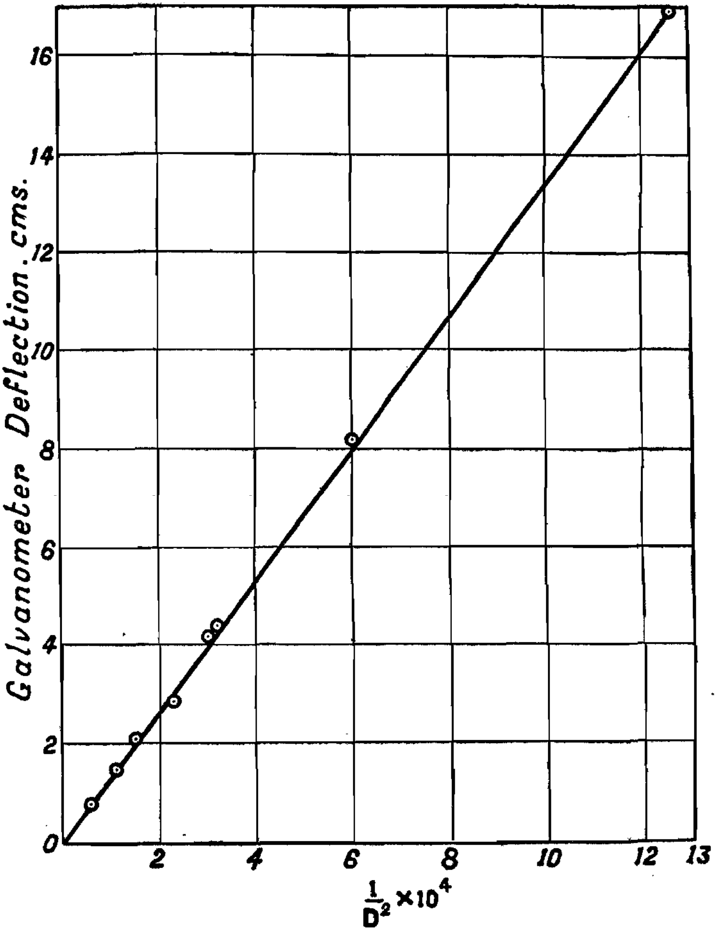
Galvanometer Deflection . cms.
16
14
12
10
8
6
4
2
0
2
4
6
8
10
12
13
$\frac{1}{D^2} \times 10^4$

FIG. 3.

to adjust with sufficient accuracy the distance between the electrodes, normally 0·5 mm. The cell was illuminated by means of a quartz mercury arc lamp working at atmospheric pressure and consuming 2·5 amps. at 92 volts.

Before recording actual measurements a preliminary inquiry was made into the effect of (1) the gas pressure in the cell and (2) the distance apart of the electrodes. Using a sample of the compound HS.Hg.CNS, the galvanometer deflection with air at 1·0 mm. in the cell was 4·9 cm., at 2·0 and 2·5 mm. it was 5·0 cm. and at 3·0 mm. it fell to 4·2 cm. The existence of a maximum value is in accordance with similar observations by Toy, Edgerton and Vick and the order of pressure is the same. In all subsequent experiments the cells were evacuated to 2·0 mm. pressure, a slight variation of pressure in this region having a negligible effect.

Measurements were next made of the photoelectric current when the distance between the electrodes was varied. An increase of the distance from 0·33 to 0·44 mm. reduced the current by 1 per cent. and at 0·59 mm. there was a further reduction of 2 per cent. These changes are almost within the limit of experimental error when all factors are taken into consideration. As far as possible the electrodes were set 0·5 mm. apart. The thickness of the coating might have some influence upon the observed current but comparative experiments indicated that this factor was negligible.

Action of light on different compounds.—Twelve phototropic mercury compounds were studied and it was found that, in general, the initial photoelectric current was small, and that on continued exposure there was an increase until a maximum was reached. Simultaneous darkening of the compound occurred. If the compound was left in the dark overnight and again exposed, the initial photoelectric current was larger than before, but the final current was approximately the same. This result could be repeated on successive days although the initial currents were somewhat irregular, according to the duration of exposure on the preceding day and the exact time allowed for recovery. If the cell was heated to 100° for some hours the initial current was reduced very nearly to that given by the fresh substance. These observations are in agreement with the hypothesis that the compounds when prepared in the dark give rise to little or no photoemission while the blackened forms have considerable activity. If this is the case it is possible to form a quantitative estimate of the relative amount of the two varieties in the surface layer and thus make more accurate estimates of the rate of darkening than is possible by judging the shade. Table I gives a few values for the initial and final galvanometer deflections on different days and illustrates the order of

accuracy which may be expected in these experiments. In order to obtain comparable results the apparatus was always adjusted so that a standard cell containing a strip of polished silver gave a fixed deflection on the galvanometer (43 cm.).

TABLE I.

Initial and final photoelectric currents at different periods.

HS.Hg.CNS (*a*)			HS.Hg.CNS (*b*)			I.Hg.CNS			I.Hg CNSe		
Day	Initial	Final	Day	Initial	Final	Day	Initial	Final	Day	Initial	Final
1	0·2	9·4	1	0·5	9·3	1	1·3	3·4	1	3·0	13·0
2	1·6	9·1	2	3·0	9·3	2	1·7	3·3	2	4·0	13·3
7	0·5	9·7	3	3·5	9·5	3	2·3	3·4	3	4·3	13·3
9	2·0	9·3	4	3·0	9·3	4	2·5	3·2	4	4·9	13·4
10	7·0	9·7	5	5·0	9·6	5	2·4	3·4	5	7·5	13·4
12	6·5	9·6				6	2·8	3·3	7	4·5	13·5
13[1]	1·5	9·3									

$2HgS.HgCl_2$			$2HgS.HgBr_2$			$2HgSe.HgI_2$			$2HgS.Hg(CNS)_2$		
Day	Initial	Final	Day	Initial	Final	Day	Initial	Final	Day	Initial	Final
1	0·1	10·5	1	6·5	14·6	1	2·0	6·2	1	0·2	3·4
2	0·6	10·3	2	9·0	14·3	2	4·3	6·0	2	0·8	3·4
3	4·6	10·6	4	7·4	14·5	3	4·2	6·3	3	1·8	3·5
4	7·8	10·5	6	7·8	14·4	4	4·8	6·0	4	2·6	3·4
5	9·0	10·6	7	9·0	14·3	6	3·0	6·1			
8[2]	1·5	10·8				7	3·8	6·4			
9	4·2	10·6				8	3·5	6·3			
10	6·0	10·7									

[1] After heating to 100° for 4 hours.
[2] After heating to 90° for 6 hours.

As already mentioned, the initial currents are somewhat irregular on successive days but there appears to be a distinct tendency towards a steady increase, particularly in the case of the compounds HS.Hg.CNS and $2HgS.HgCl_2$. This suggests that repeated illumination retards the subsequent rate of reversal in the dark. No effect of this nature was observed in the original experiments in which the rate of recovery was measured, but in these the compound was darkened only to a particular shade and not completely as in the present case. There are indications that the compound takes longer to recover after a prolonged exposure to light subsequent to complete darkening, but definite measurements have not yet been made and this aspect of the subject requires further investigation.

It will be observed that in some instances the initial current, even with the fresh substance, is by no means small. Readings taken at short intervals indicate that the initial rate of change is sometimes very rapid and is therefore not easy to determine exactly. The figures in Table I refer to values 5 seconds after exposure to light.

Figs. 4 and 5 show some typical curves obtained by taking galvanometer readings at short intervals after exposure to light. Curves i and ii are for the compound $2HgS.HgCl_2$ freshly prepared and after darkening and recovery overnight. The second curve is one that would be expected for a mixture of the fresh substance with about 10 per cent. of the darkened form. Curve iii is for a fresh sample of HS.Hg.CNS and is of a slightly unusual shape as the rate of emission increases with time instead of remaining constant as in the previous case. Curves of similar shape were obtained with this compound after darkening and recovery. Curves iv and v were obtained with the iodine compounds I.Hg.CNS and $2HgSe.HgI_2$. These differ from the preceding in that they give a definite emission immediately on exposure.

A different type of curve is given by $2HgS.HgBr_2$ (vi). The emission measured as soon as possible after exposure to light is high but is changing so rapidly that an accurate determination is not possible. The shape of the curve indicates that the initial emission might be zero. The case of I.Hg.CNSe shown separately in Fig. 5 is similar, the apparent tendency of the curve to start from zero being more marked as shown in curves vii and viii taken on consecutive days. In curve ix taken a day later, the initial bend appears to be absent. In order to study the initial effect more closely the light was reduced to nearly one-half its original intensity giving curves x and xi on consecutive days. These curves show a distinct tendency to pass through the origin and indicate the rapid formation of an intermediate compound

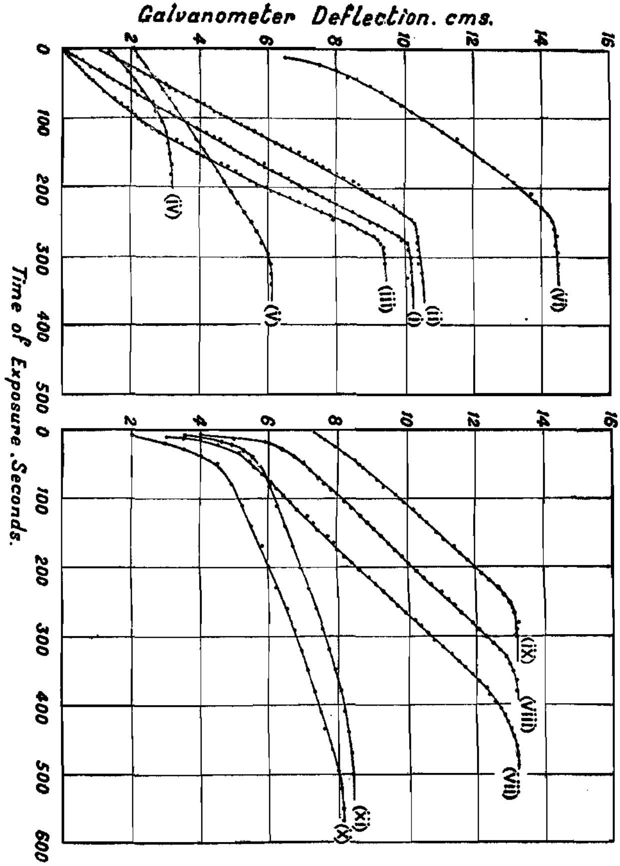

FIG. 4.

FIG. 5.

having approximately one-half the emissive power of the final product. When the first bend is reached the colour change is only slight, so that the intermediate compound appears to be of the same colour as the original one.

It is possible that action of a similar nature occurs with other compounds, but owing to the rapidity of the first change, measurements are difficult to make.

Wavelength of the active light.—Although the colour change of the compounds under investigation is greatest in the visible region, no photoelectric effect is produced by visible light. The same is the case for ultra-violet light filtered through glass or a solution of cobalt sulphate and nickel chloride transparent to 2600 A. U. It appears therefore that only light of wavelength less than this gives rise to electron emission.

It might be suspected that the ultra-violet light has some specific action on the compounds causing their activity to increase on irradiation. This was tested in the case of the substance I.HgCNSe by darkening it through a green filter and then exposing to the mercury arc. The initial emission was found to be the same as the maximum reached when exposed to the arc without a filter, so that it is the darkened form of the compound which is active irrespective of its method of formation.

A similar experiment was made with the same compound in the stage of the initial rapid change. Exposure through a green screen had the same effect as exposure to the direct arc, so that the formation of the first product is not specific to ultra-violet light.

The Relation between Light Sensitiveness and Photoelectric Emission.

As already mentioned, the green region of the spectrum is the most active in producing colour changes in these compounds while only ultra-violet light gives rise to electron emission. If there is any relation between the two properties it would most probably be found by comparing them for light of similar wavelength. In the first paper of this series it was shown that the colour change in ultra-violet light was slow, but the relation to the velocity for the visible region was not determined and it by no means follows that the ratio is constant for different substances.

We have now measured the times taken for darkening to the same shade (*a*) when exposed directly to the quartz arc (*b*) when exposed through an ultra-violet filter which cut out the visible region almost entirely and transmitted the ultra-violet as far as 2800 A. U. The results are shown in Table II and it may be seen that the ratios of the times of darkening vary from 2·0 to 4·2. Much importance cannot be attached to the relation between the figures for different compounds as the shades to which darkening took place are arbitrary and the colours vary for different compounds. It might be expected that a better comparison could be made from the curves showing the photoelectric

activity assuming that the compounds are completely darkened when the curves turn over at the top. Figures for this quantity are given in column 3 of Table II, the actual values being multiplied by a factor to make them comparable with those in column 4, light of different intensity having been employed in the two cases. It will be noted that the two sets of figures although somewhat correlated, differ in a marked manner in several cases. It is possible that most of the compounds are slightly transparent to visible light but opaque to ultra-violet light so that the photoelectric effect would reach a maximum as soon as the surface layer of molecules was converted into the darker form. Maximum darkening as judged by eye would require some time longer according to the transparency of the particles. In practice it was difficult to estimate the colour during the experiments on photoelectric emission because of the grid which covered the compound, but as far as could be seen, the colour when the maximum emission was obtained was usually somewhat lighter than the deepest shade which could be reached.

TABLE II.

Light Sensitiveness and Phototropic Activity.

	Compound	Time of darkening, minutes			Ratio		Max. photo-electric current amps. × 10^{-11}
		(a) From curves × 2·1	(b) Direct arc	(c) Through filter	c : b	c : a	
1	HS.Hg.CNS ...	9·6	6	25	4·2	2·6	15
2	Cl.Hg.CNS ...	80	95	...	...	...	1·5
3	I.Hg.CNS ...	5·0	20	63	3·2	12·6	5
4	Br.Hg.CNSe ...	8·6	30	72	2·4	8·3	6
5	I.Hg.CNSe ...	14·8	25	70	2·8	4·7	21
6	I.Hg.CNO ...	10·5	30	75	2·5	7·1	1·5
7	$HgCl_2.2HgS$ ...	9·6	12	33	2·8	3·4	16
8	$HgBr_2.2HgS$ ...	8·6	10	29	2·9	3·4	22
9	$HgI_2.2HgS$ ...	7·5	9	24	2·7	3·2	82
10	$HgI_2.2HgSe$ ...	10·5	40	84	2·1	8·0	9
11	$Hg(CNS)_2.2HgS$ ...	9·6	10	30	3·0	3·1	5
12	$Hg(CNSe)_2.2HgS$ ...	...	35	82	2·3	...	1·5
	Ag ...	...	..	...	...	...	67
	HgS ...	...	...	...	...	...	62
	HgSe ...	...	...	...	...	...	5

The times of darkening (*c*), although for ultra-violet light, are for longer wavelengths than those producing the photoelectric effect as there was no emission when the light was passed through the ultra-violet filter. The ratios to the times (*b*) for unfiltered light do not vary very widely and it is possible that darkening times for the extreme ultra-violet would be of the same relative order. The ratios *c* : *a* are much more divergent and it is difficult to say which figures more nearly represent the times of darkening. Whichever are adopted, however, there appears to be no quantitative relationship between the times of darkening and the photoelectric emission. In several cases the phototropic and photoelectric activities run parallel for related compounds, for example the chlorine compound 2 is less active than the iodine compound 3, and the activity rises from chlorine to iodine in the compounds 7, 8 and 9. The selenium compounds 10 and 12 are less active than the corresponding sulphur compounds 9 and 11, but the iodine compounds 3, 5 and 6 appear to be irregular. According to column (*c*) the selenium compound is intermediate in phototropic activity, according to (*a*) it is the least active while its photoelectric activity is the greatest of the three.

Photoelectric fatigue.—None of the substances examined showed any signs of photoelectric fatigue when exposed for 80 minutes. The photoelectric currents remained constant over this period within the limit of experimental error.

SUMMARY.

1. The photoelectric emission from 12 phototropic compounds of mercury has been examined and it is found that in all cases the emission from the compounds prepared in the dark is zero or very small.

2. On exposure to light the emission increases to a maximum and remains steady without any sign of fatigue. The maximum current was 82×10^{-11} amp. in the case of the compound $HgI_2.2HgS$ in air at 2 mm. pressure.

3. In some cases the increase in emission takes place in two stages with different rates of increase pointing to the formation of an intermediate compound.

4. On keeping in the dark or on heating, the photosensitiveness decreases very nearly to that of the freshly prepared compounds.

5. The dark varieties of the compounds give the same emission whether formed by the action of visible or of ultra-violet light.

6. There is no definite relation between phototropic and photo-electric activity although there is some correlation in the case of compounds of the same class.

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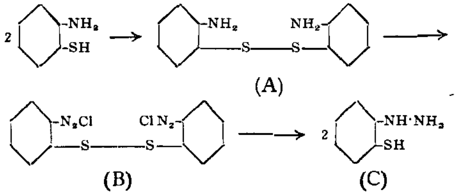
ATTEMPTS TO SYNTHESISE *Ortho*-THIOLPHENYLHYDRAZINE

By Praphulla Chandra Guha and Tejendra Nath Ghosh.

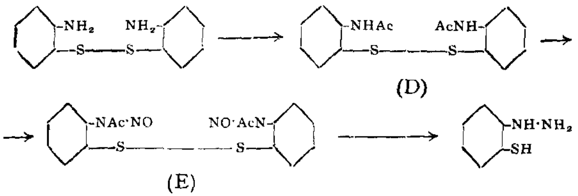
o-Derivatives of benzene, namely, dihalogen, diamino-, dihydroxy-, dithiol, aminohydroxy-, halogenamino-, thiolamino-, etc., are well known for their extensive reactivity with suitable agents to form heterocyclic compounds. The hydrazino-group also combines freely with a large number of organic types to form diverse open and closed chain compounds. *o*-Hydroxyphenylhydrazine and *o*-thiolphenylhydrazine, if obtainable, would open out a new field for investigation among heterocyclic compounds containing nitrogen and oxygen, or sulphur; accordingly, various attempts to prepare the thiolhydrazine have now been made.

o-Thiolphenylhydrazine cannot be prepared from *o*-thiolaniline by diazotisation and subsequent reduction of the diazonium group. In fact Jacobson (*Annalen*, 1893, **277**, 208) studied the action of nitrous acid upon *o*-aminothiophenol and obtained *o*-phenylenediazosulphide with a very small quantity of thianthrene. It was expected that if the tendency to ring-closure between the diazo- and mercaptanic groups were prevented by converting the thiol group into a disulphide, the resulting diaminodiphenyl disulphide might be converted into the desired *o*-thiolphenylhydrazine according to the following scheme:



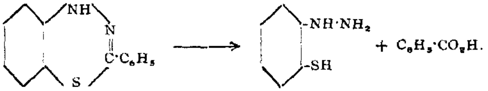
The diazonium salt (B) was found to be abnormal, however, inasmuch as it could not be reduced to the compound (C), nor could it be coupled with aniline, potassium ethyl xanthate, etc. The diazo-compound suffered decomposition on keeping or in presence of coupling and reducing agents, producing in every case a brownish tarry matter which furnished on steam distillation a pale yellow oil with empirical formula $C_{12}H_8OS_2$. It is thus evident that the diazonium compound (B) does not react with any of the above agents and is slowly decomposed in presence of water yielding the unstable *oo'*-dihydroxydiphenyl disulphide; this loses a molecule of water to yield the oxydiphenylene disulphide, $C_6H_4 \langle {}^{O}_{S_2} \rangle C_6H_4$

This is the first instance of a seven-membered ring containing one oxygen and two sulphur atoms being synthesised. Failing in the reduction of the tetrazonium salt of the disulphide (B), it was thought worth while to proceed as follows :—

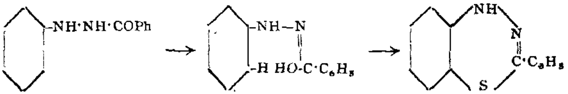


Diaminodiphenyl disulphide readily forms the diacetyl derivative (D) which, however, could not be made to yield a nitroso-derivative, though different possible methods (*Ber.*, 1876, **9**, 464 ; 1877, **10**, 959) for introducing the nitroso-group were tried (see below). This reluctance of an imino-group to yield a nitroso-derivative resembles the inert character of the diazonium salt (B), and in both cases some steric influence may be responsible.

Benzenyl-*o*-aminothiophenol yields *o*-aminothiophenol when heated with fused potash (*Annalen*, 1881, **208**, 291 ; *Ber.*, 1880, **13**, 1223 and 1887, **20**, 2259) ; it was thought advisable to apply this treatment to 2 : 3-benzo-6-phenyl-1 : 4 : 5-thiodiazine which might yield *o*-thiolphenylhydrazine thus :—



Phenylbenzothiodiazine was prepared from benzoylphenylhydrazine by heating with sulphur (compare *Ber.*, 1880, **13**, 1223, for the preparation of benzenyl-*o*-aminothiophenol).



(F)

Contrary to expectation, not only is the thiodiazine ring disrupted in fusion with potash, but the sulphur atom is eliminated by the alkali, and an aqueous solution of the fused mass yields benzoic acid and hydrogen sulphide on acidification.

EXPERIMENTAL.

Benzenyl-*o*-aminothiophenol was prepared according to the method of Hofmann (*Ber.*, 1880, **13**, 1223, footnote), the yield being 50 per cent. The disulphide of *o*-aminothiophenol was also prepared by Hofmann's method (*Ber.*, 1887, **20**, 2259) with a slight modification in the final oxidation which was more conveniently carried out by iodine solution. Yield 35 per cent.

Diazotisation and Attempted Reduction by Stannous Chloride.—*oo'*-Diaminodiphenyl disulphide (6 gms.) was dissolved in 15 c.c. of concentrated hydrochloric acid (d., 1·19) and diazotised in the usual manner with 3·6 gms. of sodium nitrite dissolved in 30 c.c. of water. To the ice-cold mixture, an ice-cold solution of stannous chloride (30 gms.) in 30 c.c. of concentrated hydrochloric acid was gradually added. The brownish yellow precipitate was allowed to stand in the freezing mixture for about an hour and then quickly filtered at the pump; otherwise the precipitate tends to become oily. On transferring it to a beaker containing water, a brownish oil separated and was distilled in steam. The light yellowish oil thus obtained was extracted with ether, dried with fused calcium chloride and freed from ether; it is insoluble in acid and alkali, possesses a peculiar smell, does not contain nitrogen and produces a reddish-pink coloration with strong sulphuric acid.

Reduction of the Diazo-compound by Sodium Sulphite.—According to the method of Fischer (*Annalen*, 1878, **190**, 71) *oo'*-diaminodiphenyl disulphide (10 gms.) dissolved in 25 c.c. of concentrated hydrochloric acid was diazotised with 5·6 gms. of sodium nitrite when a brownish yellow precipitate was obtained. To the reaction mixture was added a cold saturated solution of sodium sulphite (50 gms., i.e., 2 mols. of sodium sulphite for each amino-group). After the mixture had remained in the freezing mixture for an hour, the precipitate was very rapidly filtered by suction and washed with ice-cold water. It decomposed to a reddish-brown oil at 20–21° and was distilled in steam when tarry matters were left behind and a heavy, pale yellow oil collected; it was extracted with ether, dehydrated over fused calcium chloride, and found to be in all respects identical with the oil obtained in the previous operation.

Coupling with Aniline.—*oo'*-Diaminodiphenyl disulphide (6 gms.) was diazotised in the usual manner and an ice-cold solution of freshly distilled aniline (4·5 gms.) in 5 c.c. of concentrated hydrochloric acid was gradually added. The precipitate did not change in colour, but began to decompose at 20–22° giving rise to an oil which was purified as before and was found to be identical with the product obtained in the previous operations (Found: C, 61·84; H, 3·56;

S, 25·87. $C_{12}H_8OS_2$ requires C, 62·07 ; H, 3·45 ; S, 27·59 per cent). The estimation of sulphur in this compound by the method of Carius always gave a deficiency (see below. Compare also Ray, Guha and Das, *J.C.S.*, 1919, **115**, 1310 ; Ray and Guha, *loc. cit.*, 271 and 546).

Coupling with Potassium Ethyl Xanthate.—*oo'*-Diaminodiphenyl disulphide (6 gms.) was diazotised, the diazonium salt being quickly filtered and added to potassium ethyl xanthate (7·7 gms.) dissolved in the minimum quantity of water; the mixture was well stirred, kept in the freezing mixture for about an hour and then filtered. The precipitate gradually changed to a reddish brown oil which on steam-distillation yielded a product identical with that obtained previously (Found: C, 61·79 ; H, 3·62 ; S, 26·01. $C_{12}H_8OS_2$ requires C, 62·07 ; H, 3·45 ; S, 27·59 per cent.).

Decomposition of the Diazonium Salt.—*oo'*-Diaminodiphenyl disulphide (10 gms.) in 22 c.c. of concentrated hydrochloric acid was diazotised and after one hour in the ice-bath yielded a brownish yellow precipitate of the diazonium salt. This was very quickly filtered, washed with ice-cold water and allowed to attain the room-temperature, when it decomposed at 20–22°. The same change occurred in a beaker containing water, the resulting brownish oil yielding on distillation in steam a product identical with that previously obtained, the yield being 3·7 gms. The oil boiled and decomposed at 231°, evolving pungent fumes at 200–210° (Found: C, 61·94 ; H, 3·47 ; S, 25·18. $C_{12}H_8OS_2$ requires C, 62·07 ; H, 3·45 ; S, 27·59 per cent.).

Reduction of Oxydiphenylene Disulphide.—The oxydisulphide (1·5 gms.) was reduced with tin and hydrochloric acid and the product subjected to steam distillation. A colourless oil possessing characteristic mercaptanic properties and smell was thus obtained in quantity too small for further purification. An alcoholic solution of this oil, however, gave an insoluble yellow precipitate with lead acetate and a white precipitate with mercuric chloride. It was also soluble in dilute alkali. All these facts show that this oil is different from oxydiphenylene disulphide and is probably a mercaptan.

Diacetaminodiphenyl Disulphide.—Diaminodiphenyl disulphide (3 gms.) was heated on a sand-bath under reflux with acetic anhydride (10 c.c.) and fused sodium acetate (1·5 gms.) for 2–3 minutes only when a clear solution was obtained and immediately poured into cold water which precipitated a colourless semi-solid ; on partial neutralisation with concentrated sodium carbonate the product solidified. It was filtered, washed with acetone and ether to remove any tarry matter and finally crystallised from acetone ; m.p. 154°. Yield 3 gms. (Found : S, 19·21 ; $C_{16}H_{16}N_2O_2S_2$ requires S, 19·28, per cent.).

Attempts to prepare the Nitroso-derivative.—(1) To an ice-cold suspension of the acetyl derivative (2 gms.) in moderately strong hydrochloric acid was added an aqueous solution of sodium nitrite (1 gm.) drop by drop with constant stirring; there was no change. (2) The acetyl derivative was suspended in moderately strong hydrochloric acid and treated with the gas from strong nitric acid and arsenious oxide; in this case also the acetyl derivative was left unchanged. (3) The same gas was passed into the crystalline paste obtained by rapidly cooling in ice a solution in hot glacial acetic acid. A green coloration appeared, but the crystals did not dissolve. The clear filtrate from glass-wool was diluted with ice-cold water, and the colourless precipitate shown to be unchanged acetyl derivative.

2 : 3–Benzo–6-phenyl–1 : 4 : 5- thiodiazine (F).—Benzoylphenylhydrazine (80 gms.) was intimately mixed with sulphur (30 gms.) and heated in an oil-bath at 190–200° for about 24 hours. The black tarry mass thus obtained was transferred to a porcelain basin, heated with strong hydrochloric acid, filtered and poured into a large quantity of water which produced a yellowish flocculent precipitate. This extraction was repeated several times, the precipitate being filtered, washed with water and finally crystallised from alcohol, forming yellowish plates melting at 109°. Yield 5–6 gms. It is soluble in alcohol, acetone and acetic acid (Found: C, 68·61; H, 4·30; N, 12·09; S, 14·57. $C_{13}H_{10}N_2S$ requires C, 69·02; H, 4·42; N, 12·38; S, 14·18 per cent.).

Action of Melted Potash.—The foregoing compound (20 gms.) and powdered caustic potash (100 gms.) moistened with a small quantity of water was heated in a nickel basin until frothing and fuming ceased when the flame was removed and the fused mass allowed to cool. The reddish-brown aqueous solution of the melt was neutralised with dilute hydrochloric acid in a bath of ice-cold water, much sulphuretted hydrogen being evolved and benzoic acid precipitated with some black tarry matter. The solution did not give any precipitate with mercuric chloride, lead acetate, or iodine nor did it react with benzaldehyde, proving the absence of any compound containing the thiol or hydrazino-group.

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I.—CHARACTERISATION OF VERY SMALL QUANTITIES OF PROTEINS BY VAN SLYKE'S METHOD.*

By Nuggihalli Narayana and Mothnahalli Sreenivasaya.

In the course of our studies on animal and vegetable proteins, it became necessary to deal with very small quantities of substances, about 100 to 150 mg., a twentieth of the quantity usually employed for a macro-analysis. Thimann (*Biochem. J.*, 1926, **20**, 1190) has described a method for the determination of the Hausmann numbers in small quantities of proteins and we have extended the method for a more comprehensive characterisation of proteins by Van Slyke's method.

EXPERIMENTAL.

Digestion.—100 to 150 mg. of the protein were weighed out into a small Kjeldahl flask (4 × 30 cm.), fitted with a ground glass air condenser and digested for 36 hours with 10 c.c. of 20 per cent. hydrochloric acid. The acid was then removed by distillation in vacuo (20–25 mm.) at 40–45°. Soda-lime was placed in the receiver and a slow current of ammonia-free air was passed during the distillation to facilitate removal of the acid. A fresh quantity of ammonia-free water was added and the distillation repeated under similar conditions. The titratable acidity of the digest after distillation in the successive duplicates lay between very narrow limits. This fact is of great importance in connection with the subsequent precipitation of the hexone bases, which has been shown by Knaggs (*Biochem. J.*, 1923, **17**, 488) to depend on the concentration of the salts present in the digest of the amino-acids.

The syrupy mass was dissolved in ammonia-free water and made up to about 40 c.c. Two portions of 2 c.c. each were taken for total nitrogen, two of 10 c.c. each for the series of analyses and two of 5 c.c. each for the estimation of arginine.

Amide nitrogen.—Each of the 10 c.c. portions was neutralised with $N/2$ alkali till just pink to phenolphthalein and, after adding a drop

* Reprinted from *The Biochemical Journal*, 1928, **22**, 1135.

of the alkali in excess, the mixture was aerated for 2 hours at room temperature, air free from ammonia and carbon dioxide being passed. A few drops of amyl alcohol were added to prevent foaming. The ammonia was absorbed in 10 c.c. of *N*/70 alkali.

Melanin nitrogen.—The solution, after aeration, was carefully neutralised with *N*/5 hydrochloric acid and the melanin filtered through a small Jena glass filter covered with a thin layer of asbestos. The nitrogen in the precipitate was determined by the micro-Kjeldahl process.

Hexone bases.—The filtrate was evaporated on a water-bath to about 5 c.c., washed into a 20 c.c. stoppered centrifuge tube and made up to nearly 10 c.c. About 1 c.c. of concentrated hydrochloric acid was then added. The tube was plugged with cotton wool and autoclaved for 30 minutes at 25 lbs. pressure (Knaggs, *loc. cit.*); 3 c.c. of 25 per cent. phosphotungstic acid solution were then added and the tube was placed in a boiling water-bath until the precipitate had almost redissolved. After cooling, the tube was placed in the ice-chest for 36 hours. The precipitate was then centrifuged, and washed in the centrifuge thrice with ice-cold hydrochloric acid (1–10), using 3 c.c. each time. The clear centrifugate and the washings were passed through a small filter to recover any suspended particles. The combined precipitates were dissolved by the gradual addition of *N*/2 sodium hydroxide until the pink colour formed with phenolphthalein remained just permanent. The solution was made up to 25 c.c. and 5 c.c. were employed for total nitrogen and another 5 c.c. for amino-nitrogen and 10 c.c. for a micro-estimation of sulphur according to Pregl. From the sulphur-content the amount of cystine in the precipitate of the bases was calculated.

Arginine.—Arginine was estimated by arginase which was prepared from the liver of a ram. The enzyme preparation was found to be free from aminases and amidases which liberate ammonia from aminoacids and amides and its activity on a solution of arginine was determined; 97 to 98 per cent. of the arginine was found to be hydrolysed under the conditions which obtain in the accompanying experiments.

5 c.c. portions of the original amino-acid digest were neutralised with *N*/2 sodium hydroxide, ammonia was removed by aeration, as before and the P_H adjusted to 9·7 by adding a phosphate buffer. After addition of 0·5 c.c. of the enzyme extract and 1 c.c. of toluene, the reaction was allowed to proceed for 36 hours at 37°. Controls were run with 5 c.c. of water and 0·5 c.c. of the fresh enzyme extract. Urease

solution, corresponding to half a pellet of Dunning's preparation, was then added to the reaction mixture, the P_H being readjusted to 7. After 12 hours' reaction at 37° a saturated solution of potassium carbonate was added in slight excess and the ammonia estimated by aeration.

From a knowledge of the content of cystine, arginine and the non-amino-nitrogen in the basic fraction, the histidine and lysine contents were calculated.

Mono-amino-fraction.—The combined filtrates from the precipitation of the hexone bases were made up to 50 c.c. of which two 10 c.c. portions were used for total nitrogen and two 5 c.c. portions for amino-nitrogen. All ammonia distillations were carried out in the Parnas-Wagner modification of Pregl's micro-apparatus, and the amino-nitrogen determinations made with Van Slyke's micro-apparatus.

TABLE I.—CASEINOGEN.

Results expressed as percentages of total nitrogen.

—	(Micro) Narayana and Sreenivasaya	(Macro) Narayana and Sreenivasaya	Hoffman and Gortner (1924)	Dunn and Lewis (1921)	Van Slyke (1914)
Ammonia-N	9·90	10·12	10·20	10·49	10·27
Humin-N	1·16	1·86	1·51	2·13	1·28
Basic N :—					
Arginine-N	9·89	9·01	9·20	7·42	7·41
Histidine-N	3·88	4·16	6·26	6·01	6·21
Cystine-N	0·96	0·68	1·05	0·48	0·21
Lysine-N	7·67	8·12	8·49	9·09	10·30
N in filtrate from bases :—					
Amino-N	59·20	57·30	54·12	58·78	55·81
Non-amino-N ...	9·02	9·10	8·76	5·93	7·13
Total ...	101·68	100·35	99·59	100·33	98·61

TABLE II.—GELATIN.

Results expressed as percentages of total nitrogen.

—	(Micro) Narayana and Sreenivasaya	(Macro) Narayana and Sreenivasaya	(Micro) Thimann (1926)
Ammonia-N	1·84	1·70	2·60
Humin-N	Trace.	0·56	0·00
Basic N :—	22·61	25·04	17·60
Arginine-N	15·87	...	...
Histidine-N	2·74	...	...
Lysine-N	4·00	...	...
N in filtrate from bases :—	76·2	71·06	79·60
Amino-N	57·40	...	...
Non-amino-N	18·80	...	...
Total ...	100·65	98·36	99·8

SUMMARY.

A method for the characterisation of very small quantities of proteins by Van Slyke's method has been described.

Our grateful thanks are due to Prof. R. V. Norris for the keen interest he has evinced during the course of this work.

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[Accepted, 28–1–29.]

II.—THE DETERMINATION OF PYRUVIC ACID.*

By Basettihalli Hanumantha Rao Krishna and Mothnahalli Sreenivasaya.

The methods employed for the estimation of pyruvic acid are largely based upon the reaction of its carbonyl group with phenylhydrazine. Its estimation in complex biological fluids where there are other compounds which react likewise is therefore difficult and, particularly when pyruvic acid exists in small quantities amidst these compounds, the estimation is unreliable even when controls are run. This difficulty was greatly felt in some of our investigations, and it was thought worth while to study some of the existing methods which could be satisfactorily employed as such or modified for our purpose.

The production of an intense blue colour by sodium nitroprusside and ammonia, especially in the presence of a little acetic acid, has been claimed to be specific for pyruvic acid and unaffected by acetaldehyde.

The precipitation of pyruvic acid as hydrazone by excess of phenylhydrazine followed by the determination of the excess phenylhydrazine has been the subject of considerable study (Smedley-MacLean, *Biochem. J.*, 1913, **7**, 611, Simon and Piaux, *Bull. Soc. Chim. Biol.*, 1924, **6**, 477).

The reduction of pyruvic to lactic acid by zinc and hydrochloric acid and the subsequent estimation of lactic acid according to the method of Fürth and Charnass has been adopted by Lieben (*Biochem. Z.*, 1923, **135**, 240).

The oxidation of pyruvic to acetic acid by a slight excess of hydrogen peroxide has been shown to be quantitative by Holleman *Rec. Trav. Chim.*, 1904, **23**, 147), and potassium dichromate has been employed similarly and the excess dichromate estimated.

As it is not the purpose of this paper to give the results of the comparative study of these methods, it will be sufficient to say that none of them as such was found suitable in our experiments, and all required some modifications before they could be employed.

The necessary conditions for a method which would be suitable to our requirements were that it should be applicable to small quantities of the acid, usually between 0·5 and 15 mg., and in solutions of very low concentration, i.e. 0·1 to 0·05 per cent.

* Reprinted from *The Biochemical Journal*, 1928, **22**, 1169.

The method finally developed is a modification of Lieben's technique, and is based upon the following reactions:

$$CH_3{\cdot}CO{\cdot}CO_2H + 2H = CH_3{\cdot}CH\,(OH){\cdot}CO_2H \quad \text{.........(i).}$$

$$CH_3{\cdot}CH\,(OH)\,{\cdot}CO_2H + O = CH_3{\cdot}CHO + CO_2 + H_2O \quad \text{........(ii).}$$

The reduction of pyruvic to lactic acid by zinc and hydrochloric acid has been shown to be quantitative by Lieben. The lactic acid was estimated according to Fürth and Charnass. A micro-adaptation of Fürth and Charnass's method, with small quantities of pyruvic acid reduced by zinc and hydrochloric acid, gave very irregular results, due probably to very slight but irregular decomposition of pyruvic acid when boiled for a long time with 10 per cent. hydrochloric acid. With higher concentrations of pyruvic acid the results are of the same degree of accuracy as those of Lieben, and, with an empirical factor for lactic acid, its equivalent of pyruvic acid can be accurately calculated. Some of the results obtained according to the micro-adaptation of Fürth and Charnass's method of oxidation, and for larger amounts of acid according to the original method, are given in Tables I and II respectively. In all these cases, however, the titration of bound aldehyde was made according to Clausen (*J. Biol. Chem.*, 1922, **52**, 263).

TABLE I.

Different amounts of pyruvic acid were reduced by boiling with 50 *c.c. of* 10 *per cent. HCl and* 0·5 *to* 0·75 *g. of zinc for* $2\frac{3}{4}$ *hours.*

Pyruvic acid taken (mg.)	0·68	1·36	1·36	2·04	2·04	3·40	3·40	2·72
Found, per cent.	79·4	85·3	81·6	85·3	82·9	86·5	82·9	83·1
Pyruvic acid taken (mg.)	2·72	6·80	6·80	10·20	10·20	13·60	13·60	
Found, per cent.	84·6	85·5	82·0	86·2	88·8	89·2	87·1	

According to the same method Clausen finds with lactic acid a yield on an average of approximately 92 per cent., about the same as that found for larger quantities. For comparison some results of the estimation of larger quantities of pyruvic acid according to the original method of Fürth and Charnass are given in Table II.

TABLE II.

Different amounts of Pyruvic acid were reduced by boiling with 100 *c.c.* 10 *per cent. HCl and* 2 *g. of zinc for* $2\frac{3}{4}$ *hours.*

Pyruvic acid taken (mg.)	12·14	12·14	24·28	24·28	36·42	36·42	48·56
Found, per cent.	86·9	85·8	86·7	90·2	88·3	87·9	88·5
Pyruvic acid taken (mg.)	48·56	60·7	60·7	121·4	121·4	182·1	182·1
Found, per cent.	89·9	88·4	90·1	89·56	91·6	89·6	92·2

The values for pyruvic acid computed according to the empirical factor of Fürth and Charnass give results accurate to within about ± 3 per cent.

The irregular results obtained when small amounts of pyruvic acid are estimated were at first supposed to be due to the length of time during which they were in contact with boiling acid, and unsuccessful attempts were made to find a time during which a maximum and constant yield was obtained.

In view of this irregularity, reduction at low temperatures was tried and the use of a zinc-copper couple in sulphuric acid solution was found satisfactory. The oxidation of lactic acid was carried out by a slight modification of a recent method due to Friedmann *et al.* (*J. Biol. Chem.*, 1927, **73**, 335). In this case the yield was lower than with other methods (about 80 per cent.) but it was quite constant within a large range of pyruvic acid concentrations.

The apparatus used by us is shown in Fig. 1 and is much simpler than that of Friedmann. The transference of aldehyde to the receiver is facilitated by passing carbon dioxide from a cylinder through the apparatus. The gas is passed through a long column of saturated sodium bisulphite solution and then through water, which is frequently changed, before it enters the reaction vessel. The yield of aldehyde has

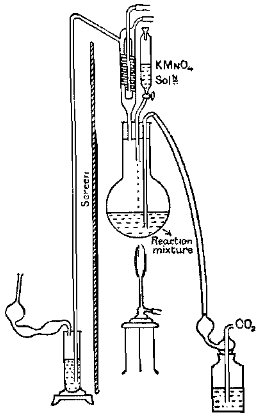

Fig. 1.

The estimation of pyruvic acid in pure solutions.

been found to depend upon the rate of aeration also, and this can be effectively controlled.

1 to 5 c.c. of solution containing 0·25 to 15 mg. of pure pyruvic acid are treated with 50 c.c. of 17·5 per cent. H_2SO_4 and 0·5 to 1 g. zinc together with 1 c.c. of 10 per cent. copper sulphate solution. After an hour the solution is filtered into the reaction flask (a 500 c.c. Kjeldahl flask) and neutralised slowly with 60 per cent. NaOH added drop by drop, dimethylaminoazobenzene being the indicator. 10 c.c. of 10*N* H_2SO_4 containing 0·1 *N* $MnSO_4$ are then added and the oxidation is carried out with 0·01 or 0·005 *N* permanganate the bound aldehyde being then titrated according to Clausen's method. The use of hydroxylamine to catch the acetaldehyde, as suggested by Leone and Tafuri (*Ann. Chim. Applic.*, 1925, **15**, 206), was tried, but the end-point was not very sharp, and in all further work Clausen's method was adopted. Some of the results of estimations of pyruvic acid in pure solution with different quantities of the acid are given in Table III. A few experiments were also conducted in which the oxidation of the reduced pyruvic acid was carried out with sulphuric acid. The yield of aldehyde obtained when 50 per cent. sulphuric acid at 140° was used according to Clausen showed that more drastic treatment was required, namely increase of the temperature to 160°–170° and the use of 60 per cent. acid, as had been found by Hill, Long and Lupton (*Proc. Roy. Soc.*, 1924, B, **96**, 455) and Ronzoni and Laurence (*J. Biol. Chem.*, 1927, **74**, 363). Some of the results obtained are given in Table IV.

TABLE III.

Analyses of pyruvic acid in pure solutions.

Pyruvic acid taken (mg.)	16·36	16·36	13·62	13·62	8·18	8·18	6·10	6·10
Found, per cent. *	99·6	99·4	101·9	97·8	99·3	99·3	100·6	100·6
Pyruvic acid used (mg.)	3·44	2·44	2·44	1·22	1·22	0·61	0·61	0·305
Found, per cent. *	97·7	97·6	97·6	102·3	98·4	103·2	100·0	100·0

TABLE IV.

Comparison of the permanganate and sulphuric acid methods of oxidation.

Pyruvic acid taken (mg.)	8·43	8·43	5·06	3·37	0·61	0·305	1·69
Permanganate method, found, per cent.	80·4	82·3	80·3	78·9	80·3	82·6	76·3
Sulphuric acid method, found, per cent.	80·9	80·7	81·0	82·8	78·7	78·7	78·0

The results of the estimation of pyruvic acid in pure solution according to both the methods of oxidation, showed that there is a

* Using factor (see p. 46.)

yield of 80 per cent. aldehyde, constant over large variations in the quantities of pyruvic acid used. This does not appear to be due to a factor in the oxidation of lactic acid since pure zinc lactate solutions could be determined by the same method with an accuracy of 96·2 to 97·9 per cent.

The actual losses of pyruvic acid appear to occur during the initial reduction process, probably due to the formation, along with lactic acid, of products which do not yield bisulphite-binding compounds during the subsequent oxidation. Ronzoni and Laurence (*ibid.*) in connection with their experiments on the loss of lactic acid in solution, drew attention to an early observation of Dakin, who found that lactic acid solutions on standing yielded methylglyoxal. They moreover found that a solution of zinc lactate on standing for several days gave smaller and smaller yields of aldehyde day by day until after 4 days the yield stood constant at about 84 per cent. for several weeks. This fall was noticed even when the solution was sterile and in the presence of mercuric chloride. We ourselves have observed such a loss of aldehyde, but the fall, though considerable, was not so rapid as that found by Ronzoni and Laurence. It was also found that zinc lactate solution, treated in the same way as pyruvic acid in our experiments, gave lower yields of aldehyde.

Another source of loss of lactic acid before oxidation appears to be due to the fact that it is kept in contact with sulphuric acid, and this loss increases with the time of contact: 23·3 mg. of zinc lactate were made up to 100 c.c. with 20 per cent. sulphuric acid; 20 c.c. of this solution were taken at intervals of 1½ hours and the lactic acid was estimated. The results are given in Table V.

TABLE V.

Time during which lactic acid was in contact with sulphuric acid (hrs.)	0	1½	3	4½	6
Aldehyde yield, per cent.	97·6	96·5	95·4	94·2	93·1

Taking all these observations into consideration, we are led to imagine that the small yield of aldehyde in our case may be due to the same cause as that which lessened the yield of aldehyde with lactic acid solution on standing. The possibility exists of the formation of methylglyoxal from pyruvic acid if the carboxyl group is attacked, along with the carbonyl group, through the vigorous reducing action of the zinc-copper couple, although the formation of methylglyoxal is not known to occur from reduction of pyruvic acid in practice.

Since in the estimation of pyruvic acid by this method the errors due to reduction or oxidation are quite regular over a large range of concentration of pyruvic acid, the particular drawback of a small yield

of aldehyde and the consequent necessity of using a large factor to compute the actual amount, cannot materially diminish the value of the method for the estimation of small quantities of pyruvic acid. This is shown by calculating the probable error of a single determination by the formula

$$e = \pm 0{\cdot}6745\sqrt{\frac{d_1^2 + d_2^2 + d_n^2}{n-1}},$$

where d represents the deviation of an observation from the mean of the series and n the number of observations. The probable error of the mean (e_m) is given by the equation

$$e_m = \frac{e}{\sqrt{n}}.$$

The probable error from a series of 15 determinations on the same quantity of pyruvic acid is shown in Table VI, and also the probable error of the mean.

TABLE VI.

10·18 mg. of pyruvic acid were reduced by 40 c.c. of 17·5 per cent. H_2SO_4 plus 0·75 g. Zn. and a trace of copper sulphate.

Amount of acid taken (mg.)	Amount found (mg.)	Deviation from the mean (d)	d_2
10·18	8·10	−0·04	0·0016
...	8·18	+0·04	0·0016
...	8·04	−0·10	0·0100
...	8·10	−0·04	0·0016
...	8·03	−0·11	0·0121
...	7·92	−0·22	0·0484
...	8·27	+0·13	0·0169
...	7·90	−0·24	0·0576
...	8·06	−0·08	0·0064
...	7·95	−0·19	0·0361
...	8·14	+0·00	0·0000
...	8·00	−0·14	0·0196
...	8·29	+0·15	0·0225
...	8·06	−0·08	0·0064
...	8·16	+0·02	0·0004

Mean = 8·14
$\sum d^2$ = 0·2412
therefore e = 0·0885
and the error of mean (e_m) = 0·0237.

The probable error of a single determination is, therefore, about 1·1 per cent. The mean of this series is very nearly the same as that obtained from the determination with different quantities of pyruvic acid (Table III).

Thus, if an empirical factor covering the regular error be taken, the exact amount of pyruvic acid can be computed. This factor works out to about 20 per cent., i.e. 1 c.c. *N*/10 iodine represents 5·5 mg. pyruvic acid. The values calculated on this empirical basis for various quantities of pyruvic acid have been given in Table III.

APPLICATION OF THE PROPOSED METHOD TO BIOLOGICAL FLUIDS.

The question of the specificity of the reaction upon which the proposed method of estimation is based arises when the method is applied to biological fluids. It will be seen that not only hydroxy-acids, carbohydrates and acetone but also lactic acid and acetaldehyde interfere with the determination and must be removed from the solution before pyruvic acid is estimated. The separation of pyruvic acid from other bisulphite-binding compounds is not easy, and the results of titration cannot be taken as specific unless acetaldehyde from pyruvic acid only be taken as a measure of that acid. During the usual processes for the removal of carbohydrates or proteins from biological materials, these interfering agents are not removed. It is found that pyruvic acid bound by sodium bisulphite is not extractable by ether, whereas lactic acid and other hydroxy-acids are easily extracted. Therefore the pyruvic acid may be extracted along with lactic acid and other interfering substances by a preliminary ether extraction which separates them from carbohydrates also. The ether extract with a small quantity of sodium bisulphite solution is again extracted with ether, when the pyruvic acid, which is combined with bisulphite, is separated from lactic acid, β-hydroxybutyric acid and other interfering substances.

Thus, since ether extraction was used for the separation of pyruvic acid from carbohydrates and lactic acid, there was no necessity to adopt Van Slyke's method (*J. Biol. Chem.*, 1917, **32**, 455) for the removal of carbohydrates from solution. It was moreover found that considerable amounts of pyruvic acid are lost in that process.

We had therefore to study only the separation of proteins from dilute pyruvic acid solution. Experiments were carried out with caseinogen and haemoglobin solutions to which small quantities of pyruvic acid were added. In the case of body fluids, it is found that the method of Folin and Wu (*J. Biol. Chem.*, 1919, **38**, 81) can be employed. For the separation of proteins from yeast culture solutions,

precipitation by alcohol is sometimes preferable. The solution is rendered faintly acidic and 15 to 20 times its volume of 99 per cent. alcohol added. The solution is kept overnight, filtered and the precipitate washed with 98 per cent. alcohol.

An aliquot portion of the solution after the precipitation of proteins, either by the method of Folin and Wu or by the use of alcohol, is neutralised and evaporated under reduced pressure at 40° to 50° almost to dryness. The residue is then transferred with a small quantity of saturated ammonium sulphate solution to the ether extractor, the solution rendered acid to methyl red, and the pyruvic acid extracted. The ether is evaporated and the pyruvic acid is bound by sodium bisulphite, and the solution is extracted again with ether to remove lactic acid, etc. It is estimated as before and the exact amount calculated by means of the empirical factor.

TABLE VII.

Recovery of pyruvic acid from protein solutions.

Pyruvic acid added (mg.)	Protein solution per cent.	Method of precipitation	Pyruvic acid recovered, per cent.
4·61	2 hæmoglobin	Folin and Wu	99·2
4·61	5 ,,	,, ,	98·9
4·61	2 ,,	Alcohol	99·35
4·61	5 ,,	,,	98·9
7·69	3 ,,	Folin and Wu	99·45
7·69	3 ,,	Alcohol	100·1
15·37	5 ,,	Folin and Wu	99·48
15·37	5 ,,	Alcohol	97·8
3·05	2 caseinogen	Folin and Wu	98·4
3·05	2 ,,	Alcohol	97·7
6·10	3 ,,	Folin and Wu	98·5
6·10	3 ,,	Alcohol	100·2
15·25	3 ,,	Folin and Wu	99·2
15·25	3 ,,	Alcohol	98·3
15·25	5 ,,	Folin and Wu	97·9
15·25	5 ,,	Alcohol	97·6

From such dilute solutions of pyruvic acid a recovery of 97 per cent. is therefore possible after protein precipitation.

The estimation of pyruvic acid in biological fluids may therefore be carried out as follows. The solution, which should contain not more than 15 mg. of pyruvic acid during its reduction to lactic acid, is taken and the proteins separated by either of the processes detailed above. Suppose the solution contains 0·05 per cent., about 2 to 5 c.c. of this is used for protein precipitation and the whole of the filtrate is taken up for subsequent processes. The filtrate is rendered neutral to litmus and evaporated under diminished pressure at 40° to 50°. In this process much of the preformed acetone, acetaldehyde or other volatile compounds, escapes. The substance is then transferred to a Clausen or Meyerhof extractor with a small quantity of saturated ammonium sulphate solution, rendered slightly acid and extracted with ether to separate the carbohydrates from the ether-extractable substances. The ether should have been distilled over bisulphite, as it sometimes contains bisulphite-binding compounds as impurities. The ether extract is evaporated to dryness and shaken up with a quantity of sodium bisulphite three or four times in excess of the expected amount of pyruvic acid. It is then transferred again to the ether extractor to separate pyruvic acid from lactic acid, β-hydroxybutyric acid, phenols, etc. The residue is transferred to a 100 c.c. flask and the pyruvic acid reduced by sulphuric acid and zinc with a trace of copper. The lactic acid is estimated as already described.

The errors introduced by the presence of acetone bodies is considerably diminished by the two evaporations. But even then the error due to acetone formed during oxidation, or held tenaciously, can be determined after the removal of aldehyde by Schaffer's method (*J. Biol. Chem.*, 1908, **5**, 211) as adopted by Clausen as a correction for his lactic acid determination. The titre from this distillation is subtracted from the previous titre, and after proper corrections are applied the pyruvic acid is calculated using as factor 1 c.c. of *N*/100 iodine = 0·55 mg. pyruvic acid. In many cases, except where the determination is carried out with yeast culture solutions, this determination of acetone bodies after final iodine titration is unnecessary, as the error is small. The results of the recovery of pyruvic acid added in small quantities to biological fluids, such as the body fluids of the lac insect, sheep's blood and yeast culture solutions, are given in Table VIII. The probable error of a single determination in a series, calculated from a number of experiments as before, is shown in Table IX.

TABLE VIII.

Recovery of pyruvic acid added to biological fluids.

Medium	Amount added (mg.)	Iodine titre corrected for blank and titre (c.c.)	Control (c.c.)	Amount recovered using the empirical factor	Error
Sheep's blood, 5 c.c. ...	1·51	5·5 (0·005 *N*)	0·10?	1·49	—1·3
	3·02	11·3 (0·05 *N*)	0·10?	3·08	+2·0
	6·04	11·15 (0·01 *N*)	0·10?	6·08	+0·7
Yeast culture solution, 5 c.c. ...	1·51	5·65 (0·005 *N*)	0·25	1·49	−1·3
	3·02	11·12 (0·05 *N*)	0·25	2·99	—1·0
	6·04	10·96 (0·01 *N*)	0·25	5·89	−2·5
Body fluid of lac insect, 5 c.c. ...	1·51	5·76 (0·005 *N*)	0·20	1·53	+1·3
	3·02	11·02 (0·005 *N*)	0·20	2·98	−1·5
	6·04	10·86 (0·01 *N*)	0·20	5·87	—2·9

TABLE IX.

4·48 mg. of pyruvic acid were added to 5 c.c. of the body fluid of the lac insect.

Exp. No.	Amount of pyruvic acid calculated (mg.)	d	d^2
1	4·44	+0·05	0·0025
2	4·30	−0·09	0·0081
3	4·38	−0·01	0·0001
4	4·51	+0·12	0·0144
5	4·35	−0·04	0·0016
6	4·32	−0·07	0·0049
7	4·27	−0·12	0·0144
8	4·56	+0·17	0·0289
9	4·46	+0·07	0·0049
10	4·36	−0·03	0·0009

Mean = 4·39.

Probable error e = 0·063 ; i.e. about 1·4 per cent.

SUMMARY.

1. A new method for the estimation of pyruvic acid in small quantities has been described which is claimed to be more specific than the existing methods when applied to biological fluids.

2. The probable error of a single determination has been determined.

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I.—STUDIES ON SOIL ACTINOMYCES. PART I. INTRODUCTION.

By V. Subrahmanyan and Roland V. Norris.

Although, during recent years, a considerable amount of work has been done on the *Actinomyces* occurring in the soil, our knowledge of their exact nomenclature, systematic position among micro-organisms, mode of occurrence, distribution in the soil and general physiological activity, as related to soil conditions and plant growth, still remains comparatively obscure.

The name *Actinomyces* has been used as a generic designation for these organisms by the great majority of authors: but many workers have referred to them as *Discomyces*, *Leptothrix*, *Coniomycetes*, *Nocardia*, *Streptothrix Cohn* (or *Cohnistreptothrix*), *Oospora*, etc. (*vide* Breed, and Conn, *J. Bact.*, 1919, **5**, 585; 1920, **6**, 489). Wright's adoption (*J. Med. Res.*, *N.S.*, 1904–5, **8**, 349) of the terms *Actinomyces* for the anaerobic sporeless forms and *Nocardia* for the aerobic conidia-formers, which has found acceptance by some bacteriologists, suffers from the use of distinctions which are now known to vary to a considerable extent with nutrition and are, therefore, not reliable.

Among the leading systematic biologists, Bergey and co-authors (Determinative Bacteriology, 1923) and Buchanan (General Systematic Bacteriology, 1925) classify these organisms with the bacteria; Dreschler (*Bot. Gaz.*, 1919, **67**, 65 and 147) with true fungi and Lehmann and Neumann (Atlas und Grundriss der Bacteriologie, 1920) as a separate group possessing characteristics intermediate between those of bacteria and fungi. Since all these authors based their classifications on almost identical morphological characteristics as shown in culture media, it may be inferred that such distinctions are not sufficient. Waksman (Principles of Soil Microbiology, 1927) has adduced a certain amount of evidence, on physiological grounds, to show that as a genus, *Actinomyces* are distinct from bacteria and fungi. More data of a similar type are, however, required to place the organisms in a definite, systematic position.

The mode of occurrence of *Actinomyces* in the soil is still not well defined. Our knowledge as to whether these organisms occur exclusively in the form of conidia or as vegetative mycelia interspersed with conidia, though exceedingly important from the point of view

of assessing their real importance in the soil is still inexact. Conn (*Soil Sci.*, 1922, **14**, 149) stated that by his direct staining method the presence of the vegetative mycelia of *Actinomyces*, together with those of fungi, can be demonstrated; but since the staining does not distinguish between the filaments of the two types of organisms, the evidence for their mixed occurrence, thus obtained, is hardly satisfactory. It is necessary to adduce more rigorous proof to demonstrate the exact condition in which *Actinomyces* occur in soil.

Although it has been noted by many workers that *Actinomyces* form quite a large proportion of the colonies appearing on bacterial plates, no attempt has been made to standardise a readily reproducible method that would help in the accurate enumeration of the organisms, vegetative or otherwise. The methods used by the previous workers have not all been identical, so that their results are not really comparable with each other. Other conditions being comparable, the numbers of the organisms being the index of their ability to bring about in the soil the changes characteristic of their species, the necessity for a standard-count medium can hardly be over-emphasised.

A large number of species of *Actinomyces* have been isolated from the soil and their cultural and other characteristics studied by Krainsky (*Zentr. Bakt.*, II, 1914, **41**, 639) and Waksman and Curtis (*Soil Sci.*, 1916, **1**, 99; 1919, **8**, 71), and others: but their exact mode of distribution in the soil, their respective numerical fluctuations with season and general climatic conditions, mechanical composition, physical conditions and reaction of the soil still remain to be determined in proper detail.

Hiltner (*Jahrb. Ver. angew. Bot.*, 1907, **5**, 200) and later Bright and Conn (*N. Y. Agr. Exp. Sta. Tech. Bull.*, 1919, 67) have shown that there is an increase in the number of *Actinomyces* in the soil following the addition of organic manures or treatment with lime and mineral fertilisers. The exact mode of response to such treatments by the different species and their relation to the types of crop raised or to the natural vegetation occurring on the soil has yet to be determined.

Lieske (Morphologie und Biologie der Strahlenpilze, 1921) made some study of the effect of temperature, desiccation, different types of light rays, certain chemical poisons, dyes, etc., on the *Actinomyces* of the soil: Munter (*Zentr. Bakt.*, II, 1916, **44**, 673) observed the effects of salts of different concentrations in culture media. Their investigations, however, require to be followed up, in intensive detail, on the soil itself and with reference to the species which are noted to be active in the soil.

In the course of their cultural studies Krainsky (*loc. cit.*) and Waksman (*Soil. Sci.*, 1919, **8**, 71) have shown that many soil *Actinomyces* secrete cellulases, amylases, proteases and other enzymes. More detailed and quantitative data in regard to the enzymic activities of the organisms are however, required before their physiological activity in the soil can be properly defined.

The influence of air supply on most forms of *Actinomyces* has not yet been definitely established. Since the time when Beijerinck (*Zentr. Bakt.*, II, 1900, **6**, 2) described the whole genus as being facultative anaerobes, many opinions have been expressed by different authors, some describing them as being aerobic, some as being strictly anaerobic, and so forth. Since aerobic and anaerobic conditions prevail side by side in the soil, the subject requires careful revision with quantitative studies of the different metabolic products of the organisms.

Krainsky (*loc. cit.*), Waksman (*J. Bact.*, 1919, **4**, 307) and others have studied extensively the utilisation of different carbohydrate and allied materials by *Actinomyces* for their growth and development, but information is not yet available concerning the quantities of the different compounds thus assimilated, the manner in which various substances are taken up, and the different metabolic products that are formed. Nor is anything known regarding the extent to which the normal activity of the *Actinomyces* may be modified by the other organisms present in the soil.

The investigations of Mace (*Compt. rend.*, 1905, **141**, 147), Munter (*Zentr. Bakt.*, II, 1914, **39**, 561), Waksman (*J. Bact.*, 1920, **5**, 1) and others have shown that *Actinomyces* can decompose proteins into amino-acids and ammonia; but it is not known whether they bring about similar changes in the soil where vast numbers of different types of organisms possessing similar properties are present. The extent to which *Actinomyces* can assimilate and transform mineral matter under different conditions in the soil, likewise remains unknown.

The part played by soil *Actinomyces* in the decomposition of cellulose materials in the manure heap has not, so far, been determined, although the musty odour characteristic of their presence is frequently quite pronounced, particularly when such material is stored in the dark. Perhaps the most characteristic thing about the soil *Actinomyces* is this earthy odour; but little is known about the nature of the odoriferous principle, its relation to the physiological activity of the organism or any effect it may have on the growth and development of the other organisms occurring in the soil. While it is generally recognised that carbohydrates are utilised in its production, it is a common experience that the intensity of odour may vary considerably when the organisms

are grown on different types of carbohydrate material, e.g., from the very faint odour obtained on certain sugars, to the oppressive intensity obtained with starches, suggesting that the chemical structure of the carbohydrate supplied is a determining factor in its production.

Another important physiological aspect of *Actinomyces* which merits detailed investigation is the mode in which the numerous pigments characterising the different species are produced. Conn (*N. Y. Agr. Exp. Sta. Tech. Bull.*, 1921, 83), Waksman and Curtis (*Soil Sci.*, 1916, **1**, 99), Lieske (*loc. cit.*) and others have made interesting observations on pigment formation in different culture media, but no definite knowledge of the subject is yet available. Beijerinck (*Zentr. Bakt.*, II, 1900, **6**, 2) and others have suggested various types of reactions such as quinone-formation, oxidase-action, etc., as representing the mechanism of the pigment-production, but much further work is required to arrive at any definite conclusion on the subject. The relation of the chromogenic principles to the physiological activities of the *Actinomyces* also deserves careful study.

Actinomyces are known to occur in the air, on butter (Jensen, *Zentr. Bakt.*, II, 1902, **8**, 171) and various other materials of plant and animal origin. Though it is probable that many of these forms have been derived from the soil, it is not definitely known whether such varieties exhibit the same physiological characteristics, nor is it known whether a normal soil saprophyte can, under other conditions, become an animal parasite.

The foregoing and allied problems are under investigation in these laboratories and will form the subjects of future communications.

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II.—STUDIES ON SOIL ACTINOMYCES. PART II.

Their Mode of Occurrence in the Soil.

By V. Subrahmanyan.

The investigations of Hiltner (*Jahrb. Ver. angew. Bot.*, 1907, **5**, 200), Conn (*Jour. Amer. Soc. Agron.*, 1913, **5**, 218), Krainsky (*Zentr. Bakt.*, II, 1914, **41**, 649), Waksman and Curtis (*Soil Sci.*, 1916, **1**, 99) and others have shown that the numbers of soil *Actinomyces* appearing on plates can vary according to the conditions obtaining in the soil. Although not indicating whether the organisms at the time of counting occurred as filaments or as conidia their results suggest that the *Actinomyces* must have existed in the vegetative condition for a brief period at least.

Conn (*Soil Sci.*, 1922, **14**, 149) by his direct staining method was able to demonstrate the presence of filamentous forms in soil in which young grass was growing and, associating this observation with his previous plate-counts of *Actinomyces* from the sodden soil, inferred that these filaments belonged to the group of ray fungi. But he did not prove the absence of fungal filaments which, in many cases, appear identical with those of *Actinomyces*, and which might have accounted for all the stained hyphae noticed by him. Waksman, by his direct inoculation method (*Soil Sci.*, 1922, **14**, 153) demonstrated the presence of vegetative fungi in the soil, but not the freely occurring hyphae of *Actinomyces*.

The determination of the exact condition in which *Actinomyces* occur in the soil is of great importance in assessing the value of the part played by the organisms in various phenomena. Thus, if a certain form of readily decomposable matter be added to the soil, the *Actinomyces* if present in the vegetative condition, will compete with the other organisms and utilise it for effecting the different changes characteristic of them but, if present exclusively as spores, the bacteria and other forms would use up the material present before the *Actinomyces* could germinate, while if present partly in the vegetative and partly in the spore condition, they will play a part intermediate between the two cases described above. The object of the present investigation was to define the exact condition in which *Actinomyces* occur in the soil.

EXPERIMENTAL.

A preliminary series of trials was carried out, with pure strains of *Actinomyces*, to determine whether the hyphae and the conidia could be identified as such when occurring either free or mixed with the soil.

Two common soil forms, *A. chromogenus* (Gasperini)[1] and *A. reticuli* (Waksman and Curtis), were used throughout the following series. The strains had been raised on starch-agar (Waksman, *Soil Sci.*, 1919, 8, 71) making satisfactory growth, and the cultures were about a month old at the time of usage. Growth-circlets of about 2 mm. diameter were removed with a sterile platinum needle and transferred to stoppered conical flasks each containing 200 c.c. of sterile saline (5 gms. of sodium chloride and 1 gm. of hydrated magnesium sulphate dissolved together in 1 litre of distilled water). The flasks were shaken vigorously to ensure even distribution of the broken hyphae and the conidia: the contents were then halved and while one portion, in each case, was left undisturbed, to each of the others soil (10 gms.) was added and the flasks again shaken to secure uniform dispersion.

The soil used was a surface specimen from low-lying paddy land at Dacca, Bengal, and was chosen because of its extreme uniformity and general freedom from plant residues and sundry forms of organic matter. The soil was air-dried and passed through a millimeter-mesh sieve before usage. Loopfuls of the suspensions, with and without the added soil, were examined directly under the microscope. It was observed that even with pure suspensions the conidia, which were small, short, rod-like forms as distinguished from the big, round or oval spores of fungi, were more prominent than the hyphae which, in spite of the shaking, tended to cohere and could not be readily observed. In the soil suspension neither the hyphae nor the conidia could be distinguished from the other forms of micro-organisms.

Conn's direct staining method (*loc. cit.*) was next tried. In addition to Loeffler's alkaline methylene blue, saturated aqueous solutions of six different commercial preparations of methylene blue and erythrosin were used to stain the organisms after fixing them on the slide. It was observed that the hyphae took the stains readily though they could not be obtained as evenly dispersed as their numbers would warrant. The process was repeated several times, but always with the same result. Similar trials were carried out with suspensions of two fungi, *Aspergillus niger* and *Penicillium glaucum*, but in these cases

[1] The author desires to take this opportunity of thanking the Curator of the National Collection of Type Cultures, Lister Institute, London, for his courtesy in supplying the cultures.

the stained hyphae were found to lie evenly dispersed and could, therefore, be readily distinguished from the other microflora.

Further observations showed that the actinomycetic mycelia, which were very much more interwoven than those of the fungi, could not be dispersed unless they were teased out with the needle. Such a procedure would not, of course, be possible when attempting to detect their presence in the soil. Since, it was also noticed that the actinomycetic hyphae, while generally thinner (1·6–2·3 μ), could not be readily distinguished from those of the fungi (2·1–3·8 μ) in the stained preparations, it may be inferred that Conn's method cannot be adopted satisfactorily for the detection of *Actinomyces* if they occur vegetatively in the soil. The conidia measuring 1·8–2·5 $\mu \times$ 0·8–1·1 μ did not in any case take the stains. When mixed with the soil suspension they could not be distinguished from the bacterial forms occurring therein.

Before trying Waksman's direct inoculation method (*loc. cit.*) it was felt necessary to determine (*a*) the maximum period required for the vegetative hyphae to multiply and form colonies of visible size, (*b*) the minimum period required for the spores to germinate, so that a period intermediate between the two could be chosen to distinguish the colonies originating from the hyphae from those derived from the spores.

Suspensions of *A. chromogenus* and *A. reticuli* in sterile saline with and without admixture of sterile soil were prepared in the same way as in the previous trials with the difference that the cultures were only ten days old and that the conidia were suspended separately from the mycelia. The separation was effected by removing gently, from each colony, the white, woolly portion in the case of *A. reticuli*, and the greyish, powdery crests in the case of *A. chromogenus* which contained most of the conidia, with a sterile platinum needle and washing the residual portion with sterile water until traces of the whitish or grey crests disappeared completely. The portions first removed with the needle, and the residues after washing, were suspended separately in sterile saline. Microscopic examination of the suspensions showed that the former were composed entirely of conidia, and the latter, almost completely, of fragments of mycelia. Each loopful contained many conidia in the one case and many bits of hyphae in the other; there were no blanks. Attempts to free the suspensions of hyphae from the small numbers of conidia that appeared to cling to them were not successful. Subsequent observations showed, however, that complete separation was not necessary for the germination studies.

Inoculations of suspensions were made by taking out loopfuls and depositing them on isolated spots on sterile starch-agar. Ten such

inoculations were made on each petri-dish and using three plates for each specimen, thirty trials were thus carried out in each case. The plates were incubated at 25–30° and observations on the growths of colonies made at frequent intervals. The hyphae germinated quickly, though not under twenty-four hours as the fungal hyphae were observed to do (Waksman, *loc. cit.*). Very small spot-colonies were visible after two days, but it was not until the beginning of the fourth day that the growths became distinctly visible.

Among the thirty sowings, hyphae of *A. chromogenus* were observed to have germinated in nineteen and twenty-one cases when inoculated from sterile saline and from soil suspensions, respectively. Similar counts for *A. reticuli* were sixteen and fifteen respectively. Since germinations of the same set on individual plates tended to vary from each other by one or two colonies, it may be inferred that the hyphae of both strains germinated nearly to the same extent both in saline and soil suspensions. It is, however, noteworthy that though the cultures were young, under two-thirds of the total number of inoculated hyphae could germinate. From Orskov (Investigations into the Morphology of Ray Fungi, 1923), it may be inferred that fragments from older mycelia would, under similar conditions, germinate in very much smaller numbers.

The plates inoculated with suspensions of the conidia showed no signs of germination when colonies began to appear on the other sets of plates. The first colony of *A. chromogenus* became visible only on the sixth day and that of *A. reticuli* on the seventh. It was further noticed that at the end of a fortnight, less than half the number of conidia came up on the plates, and that many had not germinated even at the end of one month, as seen from Table I.

TABLE I.

Suspending medium	*A. chromogenus*		*A. reticuli*	
	Days			
	14	30	14	30
Sterile Saline	14	14	11	13
,, ,, + Soil	12	13	11	11

The foregoing observations showed that the hyphae germinated much more quickly than the conidia on suitable media; and that following direct inoculation, colonies appearing on the plates on the fourth day suggest the presence of *Actinomyces* in the vegetative condition in the soil. In view, however, of the observation that all the bits of mycelia that were present could not come out equally well, it may be argued that the germination test will not provide a conclusive proof of the absence of hyphae in the soil: but it will show whether they are present in the active condition and can readily respond to soil treatment.

PERSISTENCE OF HYPHAE IN THE SOIL.

To ascertain whether the vegetative mycelia of *Actinomyces* can persist in the active condition in the soil for any great length of time, trials were carried out by inoculating the mycelia of each of the two strains into four different types of soil and observing their conditions from time to time. The four soils were from different provinces of India and represented the alluvial, laterite, black cotton and peaty types respectively. Air-dry specimens of these soils, after being powdered and passed through the millimeter-mesh sieve, were uniformly spread into petri-dishes in portions of 10 gms. After sterilising the dishes and their contents by autoclaving at 20 lbs. for 15 minutes, the soils were moistened with sterile tap water in quantities just sufficient to cause the particles to adhere together and to the plates, thus forming media [1] containing only the natural soils, but comparable with any artificial medium set in gelatin or agar with regard to ease of handling.

In the middle of each 'soil-plate' circlets of half-inch diameter were marked with Indian ink and sown thickly but evenly with actinomycetic mycelia. Specimens were taken out from day to day with a sterile platinum needle and examined microscopically for their respective condition. It was noticed that even at the end of three days the hyphae were observable only sparsely: on the seventh day they were hardly visible on examination either directly or after staining.

To ascertain whether the hyphae had merely split into invisible fragments, but remained viable, direct inoculations were made on the seventh day from the soil-plates into starch-agar as already described, and the growths obtained after incubation for three days noted. It

[1] Similar 'soil-medium plates' have been used by the author for various examinations of soils, and have always proved very satisfactory. Their use can be safely recommended for such chemical and biological trials as cannot be conveniently carried out with large and heterogeneous masses of soils.

was observed that on nineteen out of the twenty-four plates examined there were no colonies at all: the remaining plates carried only one to three apiece, the average per plate being less than 0·3 of a colony. Although the direct examination threw no light on the changes undergone by the inoculated hyphae, yet the germination studies showed clearly that the bits of mycelium, even if they existed as such, could no longer germinate as they would have done when transferred from one nutrient medium to another.

To determine whether the organisms remained viable in the form of conidia, the plates were incubated for a fortnight and the growths appearing at the end of that period noted. It was observed that there were more colonies on the plates than at the end of three days. The average was 5·0 colonies per plate, that for *A. chromogenus* (5·3) being slightly higher than that for *A. reticuli* (4·7). The results while indicating that the new colonies were probably derived from conidia, did not show whether the latter were (1) newly formed from the mycelia, or (2) merely those already present with the bits of mycelia, or (3) partly (1) and partly (2). The fact that the colonies appeared on only five out of every ten inoculated spots suggested however, that their numbers could not have been large.

To decide whether the hyphae passed into the conidial form and, if so, to estimate them quantitatively, an attempt was made to count the numbers of conidia present at different stages with the haemocytometer. In view of the difficulties attending direct microscope examination of the soil, acid-washed quartz sand (passing the 30-mesh sieve) was used in its place. The growths of the two *Actinomyces* on starch-agar were removed at the end of ten days, shaken with sterile water (10 c.c.), aliquot parts (3 c.c.) were pipetted aseptically on sterile quartz (25 gms.) in 250 c.c. conical flasks and incubated at 37°. The contents of the flasks were moistened from time to time with sterile water to prevent their drying. At four days' intervals the flasks were taken out, the volumes of suspensions made up to 25 c.c. with sterile saline, loopfuls removed and the number of conidia counted.

In the earlier stages some difficulty was experienced in counting, owing to the small size of the conidia and their general similarity to the shorter lengths of hyphae: but, later, it was found possible to distinguish them readily from the latter by their (1) oval shape and perfectly round edges, (2) greater breadth and thicker membrane and (3) comparatively greater opalescence; all these features rendered them much more prominent than the hyphae in the counting chamber.

The periodic countings were repeated later with two other strains of *Actinomyces*, *A. scabies* (Thaxter) Gussow and *A. albosporeus Kr.* (Waksman and Curtis) after four days' growths on potato plugs. The combined results are presented in Table II.

TABLE II.

Strain	No. of conidia per c.c. × 10,000		
	0 days	4th day	8th day
A. chromogenus	214	126	84
A. reticuli	132	74	38
A. albosporeus	175	56	33
A. scabies	158	125	80

With the marked decrease in the numbers of conidia it was also found that the hyphae observable in quite large numbers initially became later more and more scarce. In the case of *A. albosporeus* they were not to be seen at all on the eighth day.

It may be inferred that (1) even the conidia did not entirely persist, under conditions of starvation in presence of moisture, (2) there was no appreciable conversion of the vegetative mycelium into conidia or, if there were, it was more than counterbalanced by the death of the conidia themselves. The degradation of the mycelia and the conidia could not have been due to plasmolysis or autolysis of the cells because the added water was never so excessive as to flood the sand. The only inference to be drawn is that the cells, under such conditions, underwent some auto-transformation in presence of air, which led to their rapid destruction. This transformation and its attendant biochemical phenomena are now being studied, and will form the subject of a future communication.

It should be noted that sand, with its (1) readiness to become wet or dry, and (2) utter lack of colloidal, organic or other mineral matter helping to sustain life, does not adequately reproduce the conditions in a normal soil. The destruction of mycelia and conidia would not have proceeded so rapidly in soil as on sand, which, no doubt, accounts for the fact that *Actinomyces*, as observed by a number of workers, are present in practically all types of soil though in comparatively smaller numbers than the bacteria. From the foregoing observations it may be inferred that while the vegetative mycelium

neither persisted nor changed into the conidial form after standing for some time with the soil, the conidia remained to some extent as such and in the viable condition, thereby helping to perpetuate the species.

EXTENDED EXAMINATION OF SOILS.

In order to confirm the evidence from the previous observations, fifty specimens representing surface and sub-soils from different parts of India and Ceylon were examined by the direct inoculation method (Waksman, *loc. cit.*).

The localities of the soils varied from equatorial to mild temperate regions. They received rainfalls varying from over 200 inches per annum to practically nil, and represented most of the commonly known types of soils, namely, peat, clay, loam (alluvial), sand, laterite and black cotton. Their reactions varied from marked acidity to high alkalinity. They carried vegetation representative of the different types of crops found in India, namely, tea, paddy, wheat, cotton, coconut, etc. Eight of the specimens were in the wet and the rest in the air-dried condition. Further details regarding the chemical composition and other characteristics of these soils in relation to their actinomycetic flora will be presented in a later communication.

The inoculations were made by placing, aseptically, lumps of soil, each about 1 cm. in diameter, on petri-dishes containing solidified starch-agar. Five such lumps were placed on each petri-dish and since two plates were allotted for each specimen of soil, ten samples were thus examined in each case. The plates after inoculation were incubated at 25–30° and examined on the fourth day for the presence of *Actinomyces.* It was observed that there were in every case dense growths of fungi round the soil lumps, overgrowing all other types of microflora and rendering the examination exceedingly difficult. It was also noticed that the lumps of soil were inconveniently large and often tended to break up readily into fragments which rolled over the plates and caused the rapid spread of fungi. To obviate the latter difficulty and to obtain large numbers of representative growths, the inoculations were made with smaller quantities of soil in a later series.

Air-dried specimens were powdered, passed through a millimeter-mesh sieve and inoculated into starch-agar plates with a platinum loop of slightly less than 1 mm. diameter. Care was taken that the inoculation was confined to selected areas of the plates. Ten such inoculations were made on each plate and two dishes were allotted for each specimen of soil. The plates were incubated at 25–30° and the growths examined on the fourth day. It was observed that though on most

plates there was no overlapping of colonies, bacteria and fungi alone grew round the soil particles. Only ten plates inoculated with five soils carried colonies of *Actinomyces*. Eight of these had only one or two colonies apiece; but on the remaining two, inoculated with specimens of a surface soil from a paddy cultivated area from Chirakkal, South India, colonies of *Actinomyces* appeared on every one of the inoculated spots and outgrew the contemporary bacteria and fungi.

Apart from the exceptional results given by this specimen, the observations confirmed the conclusions derived from the previous studies, namely, that *Actinomyces* normally do not persist in the vegetative condition in the soil. In view of the general experience that actinomycetic colonies usually appear on the plates only after about one week of incubation and the previous observation that only the conidia began germinating at that time, it may be inferred that the organisms are normally present almost exclusively in the conidial form in the soil.

ABNORMAL VEGETATION OF ACTINOMYCES IN THE CHIRAKKAL SOIL.

The early germination of *Actinomyces* from the inoculations of the Chirakkal soil suggested that the organisms must have been present in the vegetative condition in that soil. To ascertain whether abnormality of the soil led to the exceptional results, specimens were examined under the microscope with and without staining. Even superficial observation with the naked eye showed that the specimen contained large amounts of undecomposed plant residues: microscopic examination also revealed the existence of such material in a finely divided condition. The stained preparations showed the presence of certain filamentous forms, but did not, as was expected, provide any evidence identifying the organisms.

The occurrence of plant residues in large amount suggested either that (*a*) the land might have been just harvested with the result that the plant roots, straw, etc., had not had sufficient time to be decomposed, or (*b*) the soil might have been given a dressing of some organic manure, just a few days before the specimen was collected.

In view of the previous observation that the hyphae could not persist in the soil, it was essential to determine how the organisms existed in the vegetative state even after the soil had been kept for several months in the air-dried state. Since, however, the specimen contained large amounts of plant residues, it appeared probable that the hyphæ occurred on the latter. To verify this, a portion of the soil was freed from the plant residues, as far as possible, with the aid of a

hand lens, and loopfuls of it inoculated into starch-agar, on ten dishes, in the manner already described. It was observed that the counts taken at the end of three days, though varying among themselves, were distinctly lower than those obtained for the portion containing the organic matter. Only one plate carried five colonies while others contained either none, or only one to three apiece, the average being 1·9 colonies per plate.

From the above observation it may be inferred that even in the soil which gave rather abnormal evidence of *Actinomyces* existing vegetatively, the organisms did not occur in the soil itself, but on the plant residues and other forms of undecomposed organic matter which were really foreign to the soil.

Effect of Different Substances on the Condition of Actinomyces.

The previous observations while showing that in the normal soil *Actinomyces* occur mostly in the conidial form, did not prove that this would persist under soil-conditions of all types. To determine whether specific nutrients or stimulants could quicken the germination of the conidia and help the organisms to function as they would if occurring vegetatively, trials were conducted by the direct inoculation method already described, adding different types of substances to the Dacca soil used in an earlier experiment. Portions of the soil (10 gms.) were weighed out into sterile petri-dishes and the following substances (0·1 gm.) added each being dissolved, suspended, or merely shaken with sterile water (3 c.c.) according to their respective properties :—

Carbohydrates.—Xylose, arabinose, dextrose, laevulose, sucrose, maltose, lactose, raffinose, maltodextrin, starch (soluble) and glycogen ; also glycerol.

Organic Nitrogen Compounds.—Asparagine, glycine, cystine, tyrosine, indole, caffeine, strychnine, peptone, casein, gelatin.

Minerals.—Ammonium sulphate, potassium nitrate, potassium sulphate, calcium chloride, calcium sulphate, calcium dihydrogen phosphate, magnesium sulphate, potassium cyanide, silver nitrate, mercuric chloride.

Plant Materials.—Dried leaf of *Lantana camara*, Linn., rice-bran, ragi-straw, powdered paddy-husk.

Organic Disinfectants, Volatile Antiseptics, etc.—Phenol, camphor, toluene, ether, chloroform, acetone, amyl alcohol, carbon disulphide.

The added materials were distributed by dropping them evenly and allowing the soil to settle down and adhere to the dishes so as to form soil-plates of the type described already. After incubating for three days at 25–30°, inoculations were made from them into starch-agar plates; these were incubated for three days and the types of growth formed on the inoculated spots noted.

It was observed that the carbohydrates and the organic nitrogen compounds brought out predominantly certain types of bacteria, and the minerals excepting mercuric chloride, bacteria together with fungi. Inoculations from the mercuric chloride plate showed the presence of only stray bacterial colonies. The plant materials caused an increased prominence of bacteria and fungi; the volatile antiseptics and disinfectants excepting camphor, of specific bacterial types only. Camphor alone quickened the vegetation of *Actinomyces*, which appeared on seven out of the ten inoculated spots. Since, however, this substance rarely finds its way to the soil, it may be inferred that the commoner mineral nutrients, and stimulants, do not excite any unexpectedly quick response from *Actinomyces*.

The foregoing observations did not, however, preclude the possibility of the conidia germinating after about one week and then competing with the other organisms for their nutrition. They may play a very important part when materials decompose slowly, as happens in the manure heap, the rotting of which generally takes several months.

SUMMARY AND CONCLUSIONS.

1. The vegetative mycelia of *Actinomyces* perished completely or passed into some non-reproductive condition after remaining for some time in the soil. They did not appreciably pass into the conidial form.

2. Though the conidia numerically decreased considerably on standing in moist sand, there was no evidence to show that they perished completely in the soil.

3. Examination of soils in large number and variety showed that their *Actinomyces* occurred almost exclusively in the form of conidia. In an exceptional case where they were observed to be present in the vegetative condition, they were so found not on the soil itself, but on the undecomposed plant residues mixed with it,

4. Different types of microbial nutrients, stimulants, etc., did not generally quicken the vegetation of *Actinomyces* when added to the soil.

In conclusion it is the pleasant duty of the author to record his sincere thanks to Prof. R. V. Norris for his keen interest in the progress of the work and suggestive criticism.

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I.—A STUDY OF THE SYMBIOTIC FUNGUS FROM THE MYSORE LAC INSECT.

By M. Sreenivasaya and S. Mahdihassan.

Lac insects are normally associated with intracellular organisms, ellipsoidal and yeast-like in appearance. The organism is a fungus and its presence is a sign of vigorous health in the insect. Similar symbiotic associates have been recorded from time to time but the physiological function of the symbionts has not been the subject of much study. Keeble has suggested that they utilise the excretory nitrogen of the host while Portier ascribes to them a more important role, since he found that they produce reactive ketones and aldehydes on fermentation.

The organism was first isolated from the Mysore lac-insect on prune-agar medium, the reaction of which was distinctly acid to litmus. It was then grown in three series of artificial media; the first series consisting of carbohydrates, the second of nitrogenous compounds, while in the third a study was made of the necessary inorganic constituents.

In the carbohydrate series, various sugars were employed in equivalent proportions, as also starch, inulin and gum arabic. Various nitrogen compounds, amino-acids, amides, purines, nitrates etc. were used in the second series as the source of nitrogen, in such quantities that the nitrogen-content of the medium was kept constant. With regard to the inorganic constituents, the table explains itself.

After eight weeks' growth the solutions were analysed in the following manner. The solution was made up to the original volume and then filtered through a bed of previously washed and ignited kieselguhr. The residue was washed repeatedly with hot water to free it from the gummy medium and the precipitate together with the kieselguhr bed was dried in the hot air oven at 90° to constant weight, ignited and again weighed. The difference was taken as the weight of the fungus crop.

The filtrate, of which the first portions were rejected, was used for determining rotation and viscosity, the latter being measured with an Ostwald's viscosimeter at 25°, taking the usual precautions. The viscosity has been taken as a measure of the gum-content of the medium.

TABLE I.

Carbohydrate Series.

No.	Source of carbon	Yeast crop in grams	Rotation in 10 cm. tube	Viscosity at 25°
1	No Sugar ...	...	...	1·30
2	Glucose ...	74·6	...	1·38
3	Fructose ...	56·4	...	1·43
4	Sucrose ..	75·4	...	1·47
5	*Maltose* ...	118·0	...	1·46
6	Lactose ...	40·0	+ 0·25	1·33
7	Arabinose ...	24·8	...	1·38
8	Xylose	51·6	...	1·38
9	Starch	25·0	?	2·37
10	Inulin	7·4	− 0·81	1·96
11	Gum arabic ..	127·6	− 0·25 (Initial, + 1·05)	1·86

From the above it follows that among the sugars, maltose yields the highest crop: gum arabic, however, serves as well if not better. Maltose and sucrose are hardly distinguishable in yielding the highest gum-content as shown by the greatest viscosity. Lactose is not quantitatively utilised by the organism, the galactose portion of the molecule perhaps being left untouched; unfortunately the galactose medium became contaminated and this point has not yet been made clear. Inulin is not utilised by the organism, while only a part of the gum arabic appears to be attacked, the laevo-component being left untouched or appearing as a result of excretion; further work alone can decide this interesting point.

TABLE II.

Nitrogen Series.

No.	Source of nitrogen	Yeast crop in grams	Viscosity at 25°	Rotation in 10 cm tube
1	No nitrogen (not inoculated) ...	...	1·32	+ 0·95°
2	No nitrogen ...	25·5	1·36	+ 0·50
3	Glycine	130·0	2·51	+ 0·19
4	Alanine	63·4	2·10	+ 0·34
5	Leucine	56·3	2·13	+ 0·43
6	Tyrosine ...	112·3	2·15	+ 0·21
7	Asparagine ...	204·0	2·18	...
8	Acetamide ...	313·4	3·25	...
9	Urea	169·2	3·10	...
10	Uric acid ...	323·6	3·33	...
11	Hippuric acid ...	73·5	2·20	+ 0·29
12	Nitrate	153·0	2·51	+ 0·10
13	Ammonium sulphate	267·0	3·10	...

Uric acid and acetamide yield the heaviest crops of yeast, ammonium sulphate coming next. When such a diversity of compounds serve as sources of nitrogen it is difficult to draw any definite conclusion. Sugar is quantitatively utilised in the presence of asparagine, acetamide, urea, uric acid and ammonium sulphate. The greatest quantity of gum is produced with uric acid and the nitrogen series generally yield a higher gum value. The utilisation of uric acid is interesting in view of Keeble's suggestion that a function of the organism is to remove the excretory nitrogen of the host.

TABLE III.

Inorganic Constituents Series.

No.	Medium	Yeast crop in grams	Rotation in 10 cm. tube	Viscosity at 25°
1	Normal	156·8	...	1·41
2	Nitrogen free ...	25·5	+0·50	1·32
3	Phosphorus free ...	5·8	+0·93	1·31
4	Calcium free ...	168·6	...	1·35
5	Magnesium free ...	266·8	...	1·42
6	Potassium free ...	149·0	+0·12	1·37
7	Sodium free ...	166·8	...	1·39
8	Calcium excess ...	136·0	+0·16	1·43
9	Magnesium excess ...	110·6	...	1·33
10	Normal (not inoculated) ...	...	+0·95	1·30

The magnesium-free medium yields the highest crop of the fungus and, with the medium containing excess of calcium, has the highest gum-content. The absence of nitrogen, phosphorus or potassium affects the carbohydrate metabolism and the sugar is not quantitatively utilised.

A large scale fermentation was carried out to study the products, the medium being composed of:—Potassium nitrate, 20 gms., hydrogen potassium phosphate, 10 gms., sodium chloride, 5 gms., magnesium sulphate, 5 gms., glucose (commercial), 100 gms., water, 2 litres.

The time allowed for fermentation was two months. The fermented mash was filtered through a kieselguhr bed and the clear filtrate distilled on a sand-bath. The first 200 c.c. were collected and found to have the following properties :—It reduces ammoniacal silver nitrate but not Fehling's solution; it answers to the iodoform reaction and with phenylhydrazine gives a yellow precipitate. All these tests point to the presence of an aldehyde or ketone, but further work is necessary to identify the constituents.

The kieselguhr fungus crop was ground up with quartz sand and extracted with ether. A light yellow oil has been obtained, which has the property of drying, or absorbing oxygen from the air. A micro-analysis of the product will throw some light on the nature of the oil. Lastly it may be stated that the comparative bio-combustion experiments carried out with the Mysore symbionts as well as *S. Cerevisea* showed that the carbon dioxide produced by the former was only one-third that given out by the latter.

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II.—THE GOLGI APPARATUS OF FREE-LIVING PROTOZOA.

By H. S. Madhava Rao.

The occurrence of Golgi apparatus has been noted in several classes of the animal kingdom by Ludford and Gatenby. Their presence even in nucleated red blood cells such as the erythrocytes of the *Sauropsida* has been recorded by Bhattacharya and Brambell (*Q.J.M.S.*, 1925, **69**, 167). Among the protozoa, the first record of the presence of Golgi apparatus is that of Hirschler (*Anat. Anzeiger*, 1914, **47**, 289) who succeeded in demonstrating these bodies by the methods used for the metazoan Golgi apparatus; but their presence in protozoa received further confirmation only so late as 1923 by King and Gatenby (*Q.J.M.S.*, 1923, **67**, 381) who described them in *Aldelea orata*, and also by Joyet-Lavergne (*Compt. rend.*, 1923, **177**, 957) who observed them in *Adelina dimidiata* and later in some coccidia (*Ibid.*, 1924, **178**, 136; **79**, 1212) and gregarines (*Compt. rend. Soc. Biol.*, 1924, **90**, 681 and 1220). King (*Q.J.M.S.*, 1926, **70**, 147) described them in *Haplosporidium*.

The Golgi apparatus studied by Joyet-Lavergne (*Compt. rend.*, 1924, **178**, 136 and 2200) indicates a connection with the parabasal type of apparatus described by Duboscq and Grasse in flagellates (*Compt. rend. Soc. Biol.*, 1925, **92**, 154; **93**, 345; *Compt. rend.*, 1925, **180**, 477). King and Gatenby (*Q.J.M.S.*, 1926, **70**, 217) have suggested a homology between certain lipoid inclusions in *Opalina ranarum* and the Golgi apparatus. A similar observation has been made by Sokolska (*Compt. rend. Soc. Biol.*, 1927, **96**, 570) although their homology with the Golgi bodies is not yet conclusively proved. In all cases thoroughly studied, the parabasal body is found to divide at cell division. Kudd (*Archiv. Protistenk.*, 1926, **53**, 191) stated that the body arises *de novo* in *Lophomonas blattarum*, but he does not believe it to be homologous to parabasals of other flagellates. Finally the association of these bodies with the blepharoblast and centro-blepharoblast recalls the arrangement of the Golgi apparatus in relation to the centrosome in metazoa. The parabasal is found to be concerned with secretion. In *Trichomonas batrachorum* and *Tetramastix bufonis* they break off from the chromophobe and fall into the cytoplasm where they seem to be dissolved according to Grasse (*Compt. rend. Soc. Biol.*, 1926, **94**, 1012). A similar observation was made in *Trichonympha chattoni* by Duboscq and Grasse (*Compt. rend. Soc. Biol.*, 1927, **96**, 94). Causey (*Univ. Calif. Pub. Zool.*, 1925, **28**, 225) describes in *Entamoeba* what he considers to be Golgi elements, arising from food vacuoles. Therefore according to him it is to be inferred that the Golgi bodies arise *de novo* in the cytoplasm in *Entamoeba gingivalis*.

Nassonov (*Arch. mik. Anat. Entwick-Mech.*, 1924, **103**, 437) compares the ring-shaped secretory apparatus in *Chilodon* and *Dogielella* with the ring-shaped Golgi bodies found in many metazoan cells. This relationship, however, requires further confirmation since it is believed that the Golgi bodies do not appear *de novo* after cell division.

The hypotheses of Nassonov and Grasse are both very interesting and seem to be supported by weighty evidence. Until further work substantiates their results, however, they cannot be regarded as proved. Observations made on the nature of the Golgi bodies in a number of free-living protozoa form the subject of the present paper.

MATERIAL.

In order to obtain as many species of free-living protozoa as possible, the organisms were isolated from (1) Activated Sludge and (2) Soil. A large quantity of sludge was filtered through a fine sieve and then through muslin, the filtrate centrifuged for five minutes at 2,000 revolutions per minute, and the upper clear portion of the liquid decanted. The small quantity of remaining liquid contains numerous organisms.

The protozoa of the soil were isolated by inoculating the soil into the culture medium and allowing sufficient time for growth. The following medium was found to be the best:—Hay infusion, 2 c.c., agar, 1·5 gms. and distilled water, 100 c.c. The isolated organisms have been found to thrive well in malted milk also, 1 gm. being dissolved in 1,000 c.c. of water. As in the previous case the culture was centrifuged and the excess of the liquid decanted.

METHODS.

The Golgi apparatus was observed in the living cell and the observations were confirmed by the several methods used in cytological technique, of which the following were tried.

Mann Kopsch.—A small quantity of the culture solution is transferred to a glass slide, and 3 or 4 drops of Mann's corrosive osmic solution added, the slide being well washed in running water after half an hour. Then 3 or 4 drops of 2 per cent. osmic acid are added and the slide kept in a desiccator containing water; if necessary ice may be added to this. Thus a small quantity of the acid will be sufficient for a number of slides and there will be an economy in the amount of acid used; the method also avoids the use of osmic acid which has been

The slides were exposed to the osmic acid treatment from eight to twelve days, being examined every day. They were then washed for about half an hour in running water, and treated with toluene to remove the blackness from the mitochondria if they were also impregnated. The modifications suggested by Ludford, namely, completing the reduction of osmic acid at higher temperatures were also tried in addition to the Mann Kopsch Altmann combination.

Sjovall.—The organisms on the slides were fixed for half an hour in formalin (20 per cent.), washed for about 15 minutes, 2 per cent. osmic acid added and the slides allowed to remain in the desiccator containing water for about eight to ten days. They were then washed for about half an hour in running water and mounted.

Golgi.—Equal parts of 20 per cent. formalin, a saturated solution of arsenious acid and 96 per cent. alcohol were mixed and a few drops added to the slides containing the culture. Fixation was carried on for about an hour, the slides were then washed quickly and passed into 1 per cent. silver nitrate solution overnight, after which they were treated with the following mixture:—Hydroquinone, 20 gms., sodium sulphate, 5 gms., formalin, 50 c.c., water, 1,000 c.c. They were then washed and bleached in the following solution for about one minute:—Potassium permanganate, 0·5 gm., sulphuric acid, 1 c.c., water, 1,000 c.c. The slides were removed from the permanganate solution just when the colour became slightly yellowish-brown and then washed; this must be done quickly and the permanganate not be allowed to remain on the slide too long. A solution of sulphurous acid (2 to 5 per cent.) could also be used. The method was found quite satisfactory.

Cajal.—This method was modified to work well in the case of the protozoa. Uranium nitrate was omitted and thorium nitrate tried instead. After fixation the slides were immersed in 1·5 per cent. silver nitrate overnight, but the results were not so satisfactory as with the Mann Kopsch method and its modifications.

Da Fano.—Formalin (15 c. c.) and 1 gm. of cobalt nitrate were added to 100 c.c. of water and a few drops of this mixture was added to the slide; after half an hour, the slide was washed quickly and treated with 1·5 per cent. silver nitrate. Hydroquinone mixture was used for reduction. This method was more satisfactory than the previous one.

Hirschler.—The organisms were fixed in modified Champy's medium and impregnated in 2 per cent. osmic acid for about eight or ten days.

Kolatschev.—Fixing was effected in modified Champy as before and impregnation was carried on in 1 per cent. osmic acid at 30° for 40–50 hours.

Lewis.—The smears are treated with Janus green (1/50,000) and exposed to iodine vapour for a second. The Golgi apparatus is stained black instantly. The method is excellent for fresh material and also for rapid work.

Discussion.

The Golgi bodies are believed by most cytologists to be definite cytoplasmic structures, while quite the opposite view, that they are artefacts produced as a result of the fixation and staining of the cell is favoured by some (*cf.* Walker and Allen, *Proc. Roy. Soc.*, 1927, B, **101**, 468).

So far as my own observations on the Golgi bodies of the free-living protozoa are concerned I am inclined to the view that these bodies are true cytoplasmic inclusions, for a positive picture has been obtained by the various osmic and silver nitrate methods generally used in cytological technique for impregnating the Golgi bodies. They may occur as small granules or rodlets, the latter sometimes forming aggregates (*cf.* Fig. 19). These observations with regard to protozoa seem to agree with those of Ludford on the metazoan Golgi apparatus. A secretory function has been attributed to these bodies when present in the metazoan cells, especially the higher vertebrates, by Nassanov, Gatenby, Ludford, Bowen and others, but whether it is so or not in the case of protozoa, I have not yet been able to determine. In a previous paper I have shown that the mitochondria present in some soil protozoa seem to be concerned with the digestive function, and that they do not arise *de novo*, and a similar phenomenon has been observed by Horning in the infusorian *Nyctotherus*. In view of the fact that the mitochondria of the protozoa behave in the same way as they do in the other classes of animals (Horning, *Aust. Journ. Expl. Biol. and Med. Sci.*, 1927, **4**, 75) the Golgi bodies which are allied to mitochondria to a certain extent may also function in the same way among the protozoa as they do in the other animal cells. In the lower forms they occur as granules while in the higher ciliated protozoa they are found with a more organised structure in the cytoplasm. The rodlets and aggregates of these appear to be transitory stages leading finally to the more organised spherical forms.

SUMMARY.

1. A number of species of free-living protozoa have been isolated and the Golgi bodies in each rendered visible by various methods employed in cytological technique.

2. The Golgi bodies are found as granules, aggregates and spheres with a more organised structure.

3. They always occur as definite bodies in the higher ciliated protozoa.

4. They are considered from the evidence adduced in this paper to be definite cytoplasmic structures.

5. The rodlets and aggregates appear to be transitory stages between the simple granules and the spherical bodies.

In conclusion, my thanks are due to Dr. A. Subba Rao for his suggestive criticisms.

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Bangalore.

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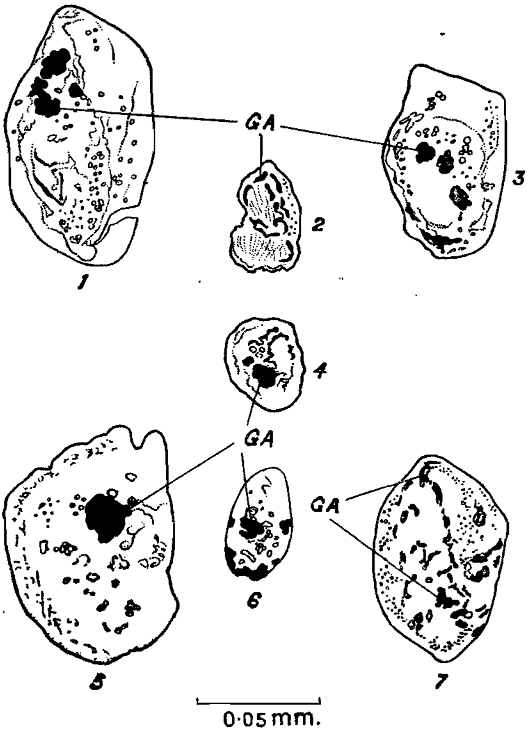
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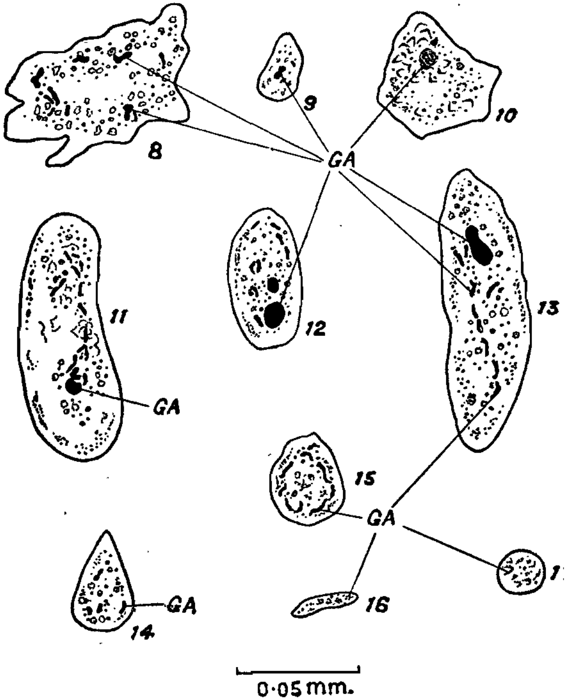

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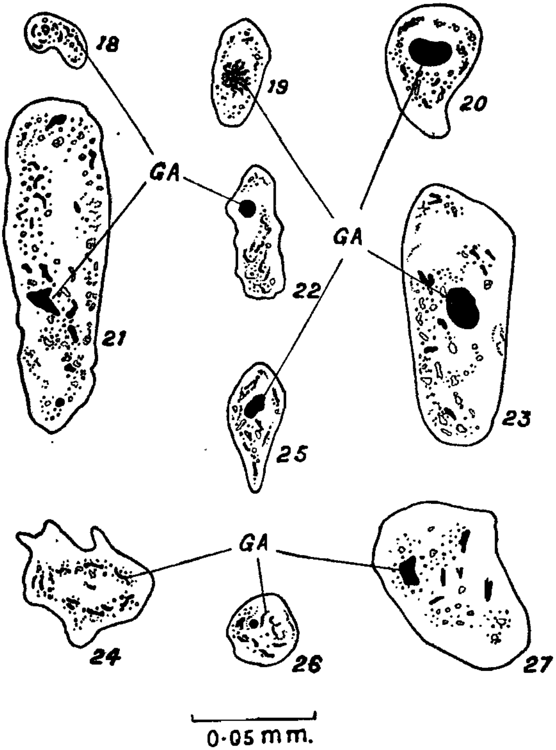
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I.—FORMATION OF HETEROCYCLIC COMPOUNDS FROM DIETHYLXANTHIC FORMIC ESTER.[1]

By Praphulla Chandra Guha and Devendra Nath Datta.

Diethylxanthic formic ester, $CO_2Et{\cdot}S{\cdot}CS{\cdot}OEt$, was prepared by Holmberg (*J. pr. Chem.*, 1905, **71**, 264) by the action of chlorocarbonic ester upon potassium ethyl xanthate. Similar compounds, *viz.*, diethylxanthic acetic ester and ethylxanthic propionic acid and their calcium, barium and sodium salts had also been prepared by the same author. This appears to be all about such di-esters and their reactions as studied up to this time. Ethylxanthic formic ester contains two active ester groups, so it was thought advisable to utilise this compound as a reagent for the construction of heterocyclic compounds. With this object in view its action upon bases like amines, hydrazines, diamines, semi- and thiosemicarbazides has been studied, and as will be seen the object has been amply realised.

The primary amines, *e.g.*, aniline, *o*-and *p*-toluidines, *m*- and *p*-nitranilines and *β*-naphthylamine react readily with the di-ester to yield the corresponding thiodicarbo-monothiodiarylamides and thus the following compounds (I–VI) have been obtained :

$$S\begin{cases}CO{\cdot}NH{\cdot}C_6H_5\\CS{\cdot}NH{\cdot}C_6H_5\end{cases}$$

(I)

$$S\begin{cases}CO{\cdot}NH{\cdot}C_6H_4{\cdot}CH_3\\CS{\cdot}NH{\cdot}C_6H_4{\cdot}CH_3\end{cases}$$

(II & III)
(*ortho* and *para*)

$$S\begin{cases}CO{\cdot}NH{\cdot}C_6H_4{\cdot}NO_2\\CS{\cdot}NH{\cdot}C_6H_4{\cdot}NO_2\end{cases}$$

(IV & V)
(*meta* and *para*)

$$S\begin{cases}CO{\cdot}NH{\cdot}C_{10}H_7\ (\beta)\\CS{\cdot}NH{\cdot}C_{10}H_7\ (\beta)\end{cases}$$

(VI)

The diarylamides form diacetyl derivatives and instead of disulphides by the oxidising action of ferric chloride, yield simple iron salts and are indifferent to the action of iodine and potassium ferricyanide. They are decomposed on being boiled with strong hydrochloric acid

[1] Reprinted from the *Journal of the Indian Chemical Society*, 1929, 6, 65.

into carbon dioxide, carbon oxysulphide, sulphuretted hydrogen and arylamines, thus :

$$S\begin{cases} CO{\cdot}NHR \\ CS{\cdot}NHR \end{cases} \xrightarrow{H_2O} S\begin{cases} CO_2H \\ CS{\cdot}OH \end{cases} + 2\ R{\cdot}NH_2$$

$$\downarrow$$

$$2H_2O : H_2S + CO_2 + COS.$$

It was expected by analogy with the action of arylamines that two molecules of phenylhydrazine would react with one molecule of the ester to yield a diphenylhydrazide. But the compound actually isolated is monothiophenylcarbazinic ester, $C_6H_5{\cdot}NH{\cdot}NH{\cdot}CS{\cdot}OEt$. So, it was assumed that the reaction had proceeded thus :—

$$\begin{matrix} C_6H_5{\cdot}NH{\cdot}NH_2 \\ C_6H_5{\cdot}NH{\cdot}NH_2 \end{matrix} + S\begin{cases} CS{\cdot}OEt \\ CO_2Et \end{cases} \longrightarrow H_2S + \begin{matrix} C_6H_5{\cdot}NH{\cdot}NH{\cdot}CS{\cdot}OEt \\ C_6H_5{\cdot}NH{\cdot}NH{\cdot}CO_2Et \end{matrix} \qquad (A)$$

and it is well known that acidyl sulphides like $S(CO{\cdot}CH_3)_2$ and $S(CO{\cdot}C_6H_5)_2$ react with ammonia, amines, hydrazines etc., to yield amides and hydrazides thus :

$$2NH_3 + S(CO{\cdot}R)_2 = H_2S + 2R{\cdot}CO{\cdot}NH_2$$

The explanation as to the course of the reaction expressed in equation (A) appears to require some modification as the other product, *viz.*, $C_6H_5{\cdot}NH{\cdot}NH{\cdot}CO_2Et$, could not be traced and also because of the fact that carbon dioxide was proved to be one product. The equation which can explain all these facts appears to be as given below :

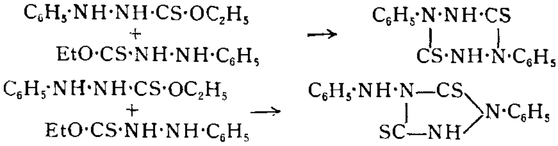
$$C_6H_5{\cdot}NH{\cdot}NH_2 + S\begin{cases} CO_2Et \\ CS{\cdot}OEt \end{cases} \longrightarrow C_6H_5{\cdot}NH{\cdot}NH{\cdot}CS{\cdot}OEt + HS{\cdot}CO_2Et$$

(VII)

The thiocarbonic ester being unstable is hydrolysed into alcohol, carbon dioxide and sulphuretted hydrogen.

By analogy with the formation of diphenylurazine by the action of heat upon phenylcarbazinic ester (*Ber.*, 1888, **21**, 2329 ; *Annalen*, 1891, **263**, 282 ; *Ber.*, 1896, **29**, 829) it was expected that phenylcarbazinic

monothio-ester, $C_6H_5\cdot NH\cdot NH\cdot CS\cdot OC_2H_5$, would yield diphenyldithio-*p*-urazine or *N*-phenyl-*N*-anilinodithiourazole, thus :

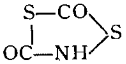


N-phenyl-*N*-anilinodithiourazole.

But the reaction was very complicated and yielded only a tarry mass. At 140–150° the ester was only partially decomposed and mostly recovered ; at still lower temperatures (110–115°) the ester was found to remain unchanged.

The action of aqueous potassium hydroxide (20 per cent.) was next studied and it was found that the ester is decomposed into phenylhydrazine, alcohol and potassium thiocarbonate from the last of which sulphuretted hydrogen was evolved during acidification.

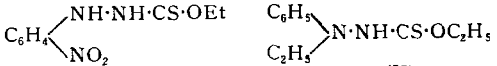
The action of strong hydrochloric acid upon the ester, $C_6H_5\cdot NH\cdot NH\cdot CS\cdot OEt$, however, led to a heterocyclic five-membered compound (VIII) containing two sulphur atoms in the ring,



(VIII)

That the two sulphur atoms in compound (VIII) form members of the ring is proved by the fact that they can neither be desulphurised with mercuric oxide nor can they yield disulphide with iodine.

The action of *p*-nitrophenylhydrazine and *unsym*-phenylethylhydrazine was also tried ; they yielded the corresponding monothiocarbazinic esters (IX) and (X) as in the case of phenylhydrazine.



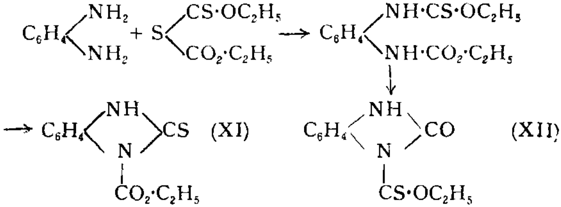
(IX) (X)

The compound (VIII) was also obtained from (IX) and (X) on treatment with hydrochloric acid.

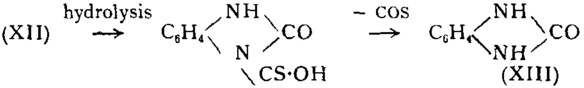
Action of Diamines.

The reaction of *o*-phenylenediamine with the ester is interesting and is attended with the formation of three compounds, *viz.*, (XI), (XII) and (XIII).

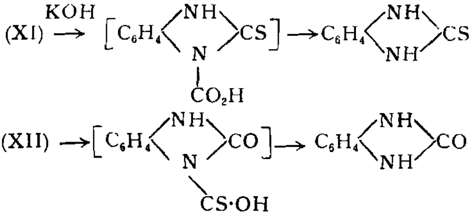
Firstly *o*-phenylene monothiodiurethane[1] is formed which under the conditions of the experiment suffers internal condensation in two different ways yielding *N*-carbethoxyphenylene thiourea (XI) and *N*-thiocarbethoxyphenylene urea (XII) thus:



The formation of phenylene urea (XIII) can be explained on the assumption that compound (XII) is first hydrolysed and then loses a molecule of carbon oxysulphide.

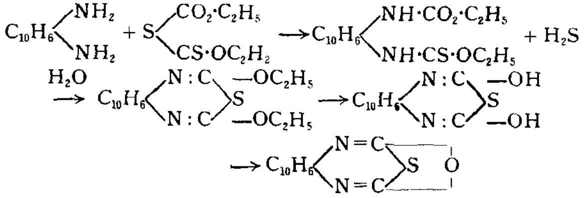


The correctness of the formulae (XI) and (XII) has been established by the facts gathered from a study of the action of potassium hydroxide solution (20 per cent.) upon them when phenylene thiourea and phenylene urea are obtained respectively from (XI) and (XII), thus:



[1] A compound of the type of (XVI) has actually been isolated with ethylenediamine.

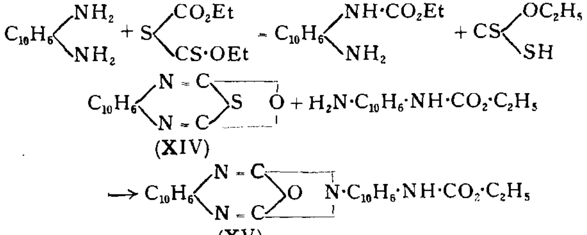
The action of 1 : 2-naphthylenediamine has also been studied but its behaviour with the di-ester appears to be a little peculiar[1] in so far as the two products finally isolated are quite different from compounds (XI, XII and XIII). Two compounds, 4 : 5-naphthylene-2 : 7-endoxy-1 : 3 : 6-thioheptadiazine (XIV) and 1-carbethoxyamino-naphthyl-4 : 5-naphthylene-2 : 7-endoxy-1 : 3 : 6-heptatriazine (XV) are formed in this case; the formation of (XIV) can be best explained thus :



(XIV)

That the sulphur atom of compound (XIV) is a member of the ring is proved by the fact that it cannot be desulphurised on treatment with mercuric oxide; moreover the absence of any group like (NH—CO) or (NH—CS) is proved by the fact that the compound is insoluble in alkali (compare compounds XI–XIII which are all soluble in alkali).

As regards the formation of the heptatriazine compound, it appears to be essential to assume, first of all, the formation of 1-amino-2-carbethoxyamino-naphthalene, $NH_2{\cdot}C_{10}H_6{\cdot}NH{\cdot}CO_2Et$, which in its turn acting upon (XIV) produces compound (XV).

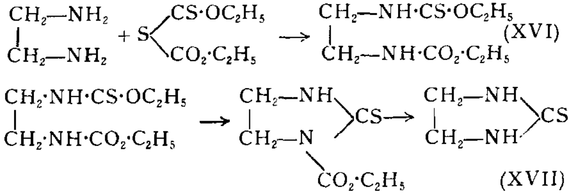


(XV)

[1] It will be seen that the experiments with phenylene- and naphthylenediamines have not been conducted under identical conditions (*vide Experimental*).

Such replacement of sulphur by a nitrogen atom by the action of ammonia and amines is well known in heterocyclic chemistry.

The action of one diamine of the aliphatic series, *viz.*, ethylenediamine has also been studied when ethylene monothiodiurethane (XVI) and ethylene thiourea (XVII) are simultaneously formed thus:



The above equation regarding the formation of (XVII) from (XVI) has been further confirmed from a study of the action of hydrochloric acid upon (XVI) when ethylene thiourea is obtained (compare the formation of phenylene thiourea from *N*-carbethoxyphenylene thiourea).

Phenylene and ethylene thiourea could up to this time be obtained only by heating *o*-phenylene disulphocyanide (Lellmann, *Annalen*, 1883, **221**, 8) and the thiocarbamic acid derivative obtained from ethylenediamine and carbon disulphide (Hofmann, *Ber.*, 1872, **5**, 242) at the temperature of boiling water.

Action of semi- and thiosemicarbazides.

Semicarbazide reacts with the di-ester to yield semicarbazide monothiocarboxylic ethyl ester, $NH_2—CO—NH—NH—CS·OC_2H_5$ (XVIII). It is interesting to note that the behaviour of thiosemicarbazide and 4-arylthiosemicarbazides towards the di-ester is different from that of semicarbazide. Semicarbazide yields the corresponding monothiocarbonic ester, whereas thiosemicarbazide and 4-arylthiosemicarbazides yield their carbonic esters.

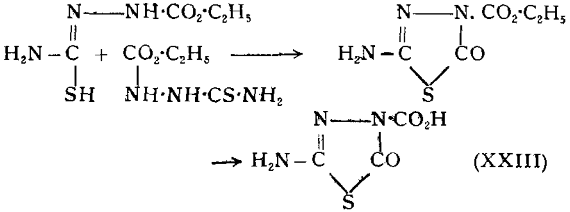
$$NH_2·CO·NH·NH_2 \rightarrow NH_2·CO·NH·NH·CS·OC_2H_5 \quad (XVIII)$$
$$NH_2·CS·NH·NH_2 \rightarrow NH_2·CS·NH·NH·CO_2·C_2H_5 \quad (XIX)$$
$$C_6H_5·NH·CS·NH·NH_2 \rightarrow C_6H_5·NH·CS·NH·NH·CO_2·C_2H_5 \quad (XX)$$
$$p\text{-}C_7H_7·NH·CS·NH·NH_2 \rightarrow p\text{-}C_7H_7·NH·CS·NH·NH·CO_2Et \quad (XXI)$$
$$C_{10}H_7·NH·CS·NH·NH_2 \rightarrow C_{10}H_7·NH·CS·NH·NH·CO_2·C_2H_5 \quad (XXII)$$

It is probable that in each case both types of compounds (*viz.*, carbonic ester and thiocarbonic ester) are simultaneously formed, of

which one preponderates. From a survey of the compounds (XVIII–XXII) a generalisation appears possible. The remote (NH_2—CO) group of semicarbazide, being more negative in character as compared with the (NH_2·CS) group of thiosemicarbazide, facilitates the formation of $NH_2 \cdot CO \cdot NH \cdot NH \cdot CS \cdot OEt$; whereas the NH_2·CS group of thiosemicarbazide being less negative, facilitates the formation of $NH_2 \cdot CS \cdot NH \cdot NH \cdot CO_2 \cdot C_2H_5$.

The action of strong hydrochloric acid upon the semicarbazide and thiosemicarbazide compounds (XVIII and XIX) is also quite interesting; the former is decomposed into alcohol, carbon oxysulphide and semicarbazide; whereas the latter yields 2-amino-4-carboxylic-5 keto-4 : 5-dihydro-1 : 3 : 4-thiodiazole (XXIII), thus:

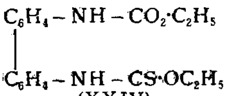
$$NH_2 \cdot CO \cdot NH \cdot NH \cdot CS \cdot OC_2H_5 \longrightarrow NH_2CO \cdot NH \cdot NH_2 + H_2O + COS + C_2H_5 \cdot OH$$



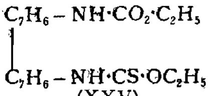
(XXIII)

It liberates carbon dioxide from sodium bicarbonate and is readily soluble in sodium carbonate showing the presence of a carboxylic group; it does not form mercaptides with mercuric chloride and lead acetate, does not form disulphide with iodine, nor can it be desulphurised by treatment with mercuric oxide. All these reactions go to show that the sulphur atom is present not in the 'SH' form, nor in the thioketonic form, 'CS,' but as a member of the ring. It forms a benzylidene derivative with benzaldehyde which shows that it contains an amino-group.

Benzidine and tolidine form with the di-ester *p* : *p'*-diphenyl-monothiodiurethane (XXIV) and *p* : *p'*-ditolyl-monothiodiurethane (XXV).



(XXIV)



(XXV)

EXPERIMENTAL.

THIODICARBO-MONOTHIODIPHENYLAMIDE (I).

Diethylxanthic formic ester (4 g.) dissolved in a small quantity of alcohol was cooled to 0° in an ice-bath and aniline (3·8 g.) gradually added. Heat was developed during the reaction with liberation of a small amount of sulphuretted hydrogen. For completion of the reaction the mixture was allowed to stand for two to three hours. It was then shaken with dilute hydrochloric acid to remove excess of aniline. A solid separated at this stage which was filtered, washed with water and crystallised from dilute alcohol, m.p. 63–64°. The substance is soluble in dilute alkali but insoluble in dilute acid. On being boiled with concentrated hydrochloric acid it decomposes into carbon dioxide, carbon oxysulphide and aniline hydrochloride (Found: N, 9·82. $C_{14}H_{12}ON_2S_2$ requires N, 9·71 per cent.).

Behaviour towards Oxidising Agents.

The substance was heated with excess of ferric chloride under reflux for about two hours and allowed to cool. The crystalline green solid was repeatedly washed with ether to remove ferric chloride (neither water nor alcohol should be used for washing, for in the former case a tarry matter is formed whilst in the latter the substance passes into solution). The residue was crystallised from dilute alcohol. The substance is insoluble in alkali but the original substance is soluble in alkali. The test for iron was made by burning the substance. A brown residue always remained which responded to the usual tests for iron. So the substance is not oxidised by ferric chloride but only its iron salt is formed. The substance was found to be quite indifferent to the oxidising action of iodine and potassium ferricyanide.

*Thiodicarbo-monothiodi-*o*-tolylamide* (II).

An alcoholic solution of *o*-toluidine (2·2 g.) was gradually added to an alcoholic solution of the ester (2 g.). As the reaction proceeded with development of heat, it had to be moderated by cooling. A small quantity of sulphuretted hydrogen was evolved during the reaction. The mixture was allowed to stand at the room temperature for two to three hours, then diluted with water and shaken with dilute hydrochloric acid to remove excess of *o*-toluidine. A solid separated at this stage which after filtration and washing was crystallised from absolute alcohol, m. p. 205° (Found: N, 8·91. $C_{16}H_{16}ON_2S_2$ requires, N, 8·86 per cent.).

Thiodicarbo-monothiodi-p-*tolylamide* (III) was obtained in a similar manner. It crystallised from dilute alcohol, m. p. 85°, is soluble in dilute alkali but insoluble in dilute acid (Found: N, 8·52; S, 20·81. $C_{16}H_{16}ON_2S_2$ requires N, 8·86; S, 20·25 per cent.).

Thiodicarbo-monothiodi-m-*nitranilide* (IV).

m-Nitraniline (1·5 g.) was added to the ester (2 g.) dissolved in alcohol. As the reaction took place with development of heat, the mixture was cooled with ice water; traces of sulphuretted hydrogen were detected. The mixture was allowed to stand at the ordinary temperature for two to three hours, diluted with water and shaken repeatedly with warm dilute hydrochloric acid to remove excess of *m*-nitraniline. The crystalline solid was filtered and recrystallised from dilute alcohol, m. p. 105°; it is soluble in dilute alkali but insoluble in dilute acid (Found: N, 14·52. $C_{14}H_{10}O_5N_4S_2$ requires N, 14·81 per cent.).

The *diacetyl* derivative was obtained by heating the substance for a short time with an excess of acetic anhydride and crystallised from alcohol, m. p. 114–115° (Found: N, 12·42. $C_{18}H_{14}O_7N_4S_2$ requires N, 12·12 per cent.).

Thiodicarbo-monothiodi-p-*nitranilide* (V).

The method of preparation and purification was the same as in the case of the previous compound. It melts at 95–96°, and is soluble in dilute alkali but insoluble in acid (Found: N, 14·65. $C_{14}H_{10}O_5N_4S_2$ requires N, 14·81 per cent.).

Thiodicarbo-monothiodi-β-naphthylamide (VI) obtained in a similar manner melts at 90°. It is insoluble in acid but soluble in alkali (Found: N, 7·05. $C_{22}H_{16}ON_2S_2$ requires N, 7·21 per cent.).

Monothiophenylcarbazinic ester (VII).

An alcoholic solution of phenylhydrazine (2·2 g.) was gradually added to the ester (2 g.) dissolved in alcohol under ice cooling when the reaction proceeded with evolution of sulphuretted hydrogen. For completion of the reaction the mixture was allowed to stand at the room temperature for two to three hours and then heated under reflux on a water-bath for half an hour more. The cold solution was then shaken with dilute hydrochloric acid to remove excess of phenylhydrazine. A solid separated which was crystallised from dilute alcohol, m. p. 73–74°. It is soluble in alkali but insoluble in acid (Found: N, 14·41. $C_9H_{12}ON_2S$ requires N, 14·28 per cent.). The filtrate from the above carbazinic ester on evaporation gave phenylhydrazine hydrochloride.

Conversion of (VII) *into* (VIII).

The ester was heated under reflux with concentrated hydrochloric acid for half an hour when a clear solution was obtained, and on cooling yielded a precipitate which was filtered, washed and crystallised from water, m.p. 135°. The substance is soluble in alkali and can be precipitated with acid. It does not give any disulphide with iodine and cannot be desulphurised with mercuric oxide (Found: N, 10·2. $C_2HO_2NS_2$ requires N, 10·37 per cent.).

Action of Heat upon Monothiophenylcarbazinic ester.

About 5 gms. of the ester were heated in an oil-bath at 230–240° for 3–4 hours when sulphuretted hydrogen was profusely evolved. A tarry mass was obtained on cooling, very soluble in ether, acetone and benzene but slightly less soluble in alcohol; it was treated with alcohol when a very small amount of solid insufficient for purification separated. The reaction was repeated at 140–150°, the period of heating remaining the same. In this case also a good quantity of tar was formed and a small quantity of the unchanged substance only could be recovered. At a still lower temperature (110–115°) for fourteen hours the whole was found to remain unchanged.

Action of Potassium Hydroxide on (VII).

The ester was heated with aqueous caustic potash (20 per cent.) under reflux for about 45 minutes. The clear solution was then allowed to cool and acidified with dilute hydrochloric acid which liberated sulphuretted hydrogen profusely and a solid containing sulphur. The substance was freed from sulphur by extracting with benzene in which the substance is very soluble. On evaporation of benzene, a solid was obtained which on examination was found to be nothing but the unchanged substance. The original filtrate contained phenylhydrazine. Evidently the monothiophenylcarbazinic ester is decomposed into phenylhydrazine, alcohol, and potassium thiocarbonate from the last of which sulphuretted hydrogen is evolved during acidification.

p-*Nitrophenylcarbazinic Monothio-ethyl ester* (IX).

p-Nitrophenylhydrazine under conditions similar to phenylhydrazine gives a similar type of compound. After crystallisation from alcohol it melts at 108–109°; is soluble in alkali but insoluble in acid (Found: N, 17·61. $C_9H_{11}O_3N_3S$ requires N, 17·5 per cent.).

Conversion of (IX) *into* (VIII).

p-Nitrophenylcarbazinic monothio-ethyl ester was heated under reflux with concentrated hydrochloric acid for half an hour when the

whole of it passed into solution. The solution was then allowed to cool when a solid separated. The mixture was then diluted with water when a part of the solid passed into solution. The residue was filtered, washed with cold water and crystallised from boiling water, m.p. 135°. The substance on analysis was found to be identical with compound (VIII). (Found: N, 10·50 per cent.).

Ethyl Monothio-ethylphenyıcarbazinic ester (X).

This was obtained similarly to the foregoing compound; crystallised from alcohol it melted at 242° (Found: N, 12·32. $C_{11}H_{16}ON_2S$ requires N, 12·50 per cent.).

Action of o-*Phenylenediamine on Diethylxanthic formic ester: Formation of* (XI), (XII) *and* (XIII).

An alcoholic solution of *o*-phenylenediamine (2 g.) was gradually added to the ester (4 g.) cooled to 0°. During the reaction, heat was developed and sulphuretted hydrogen liberated. The reaction mixture was allowed to stand at the ordinary temperature for two to three hours to complete the reaction. A solid (XI) separated at this stage which was filtered and crystallised from water, m.p. 93–94°; it is soluble in dilute acid and dilute caustic alkali but insoluble in dilute ammonia (Found: N, 11·77. $C_{10}H_{10}O_2N_2S$ requires N, 12·10 per cent.).

Through the filtrate from compound (XI) steam was passed for three to four hours to remove some oily matter; on cooling a solid (XIII) separated which was soluble in dilute alkali but insoluble in dilute acid and even in strong acid. It was crystallised from alcohol, m.p. 306° (Found: N, 21·20. $C_7H_6ON_2$ requires N, 20·89 per cent.). The compound is phenylene urea (m.p. 305°–307°).

The reaction of *o*-phenylenediamine with the ester was also conducted at the ordinary temperature without solvent. A solid separated and was shaken with dilute hydrochloric acid to remove excess of *o*-phenylenediamine. The solid portion insoluble in hydrochloric acid (XII) was filtered, washed and crystallised from dilute alcohol; m.p. 122–123°. The compound (XII) is soluble in dilute alkali (Found: N, 11·83. $C_{10}H_{10}O_2N_2S$ requires N, 12·10 per cent.).

To the filtrate from (XII) dilute ammonia was added; the precipitate on crystallisation from water melted at 93° and was found to be identical with compound (XI).

Action of Potassium Hydroxide upon (XI).

N-Carbethoxy-*o*-phenylene thiourea was heated with the alkali solution (20 per cent.) for half an hour under reflux. The solution was

then allowed to cool and acidified with dilute hydrochloric acid; the precipitate was filtered, washed with water and crystallised from dilute alcohol, m.p. 301–302°. It gives with iodine a disulphide which melts at 230°. The compound has all the properties of phenylene thiourea. The melting point of phenylene thiourea is given in Richter as 298° and in Meyer and Jacobson 292–293°. The melting point of the disulphide is given in literature as 230° (Found: N, 18·56. $C_7H_6N_2S$ requires, N, 18·66 per cent.).

Action of Potassium Hydroxide upon (XII).

N-Thiocarbethoxy-*o*-phenylene urea was heated with potassium hydroxide solution (20 per cent.) for half an hour. The solution was allowed to cool and acidified with dilute hydrochloric acid, when sulphuretted hydrogen was evolved and a white precipitate mixed with sulphur was obtained. This was freed from sulphur by dissolution in warm alcohol; on concentration of the alcoholic solution a crystalline compound (m.p. 305°) was obtained identical with phenylene urea (Found: N, 20·68. $C_7H_6N_2O$ requires N, 20·89 per cent.).

Action of 1 : 2-*Naphthylenediamine on Diethylxanthic formic ester : Formation of* (XIV) *and* (XV.)

1 :2-Naphthylenediamine (1·5 g.) was added to the ester (2 g.) under ice cooling. The reaction took place with development of heat and evolution of hydrogen sulphide. After fifteen minutes the mixture was diluted with alcohol and allowed to stand at the ordinary temperature for three to four hours during which a solid substance gradually accumulated which was filtered and crystallised from pyridine; m.p. 250°. It is insoluble in both dilute acid and dilute alkali. It is not desulphurised when heated with freshly prepared yellow mercuric oxide (Found: N, 12·21; S, 13·62 $C_{12}H_6ON_2S$ requires N, 12·38 and S, 14·15 per cent.). The filtrate from (XIV) was evaporated to dryness when a small quantity of the same substance was obtained.

The action of naphthylenediamine on the ester was tried again at a higher temperature. The reaction, as before, was first of all conducted under ice cooling in alcoholic suspension; and after an hour the mixture was heated under reflux for two hours when the reaction was completed. The solution was then cooled and the solid (XV) formed was filtered and crystallised from pyridine. The substance is soluble in no other ordinary organic solvents. It is insoluble in dilute acid and alkali; m.p. 304° (Found: N, 13·52, $C_{25}H_{18}O_3N_4$ requires N, 13·27 per cent.).

Ethylene monothio-diurethane (XVI) *and Ethylene thiourea* (XVII).

To an alcoholic solution of the ester (8 g.) ethylenediamine (2·4 g.) was gradually added under ice cooling; the reaction took place at once with liberation of heat and evolution of hydrogen sulphide. The reaction mixture was allowed to stand for two to three hours and then diluted with water when an oil separated. The dilute solution was shaken with hydrochloric acid to remove excess of ethylenediamine. At this stage the oil solidified. The solid (XVI) was separated by filtration and crystallised from dilute alcohol, m.p. 110–111°. It is insoluble in dilute acid and dilute alkali (Found: N, 12·56. $C_8H_{16}O_3N_2S$ requires N, 12·72 per cent.). To the acid filtrate from (XVI) dilute ammonia was added as in the case of the reaction with *o*-phenylenediamine but nothing was obtained.

The action of ethylenediamine on the ester was conducted without solvent and at the ordinary temperature. The solid (XVII) which separated was filtered from oil and crystallised from dilute alcohol; m.p. 193–194°. It is soluble in alkali but could be precipitated with dilute acid. It forms a mercaptide, a disulphide and a lead salt with mercuric chloride, iodine and lead acetate respectively. It gives a hydrochloride melting at 304–305° (Found: N, 27·22. $C_3H_6N_2S$ requires N, 27·45 per cent.).

To the oily filtrate from (XVII) dilute alcohol was added when it readily passed into solution which, on being diluted with water, yielded a solid substance; this was crystallised from alcohol, *m.p. 110–111°, and found to be identical with compound* (XVI).

Action of Hydrochloric Acid upon Ethylene monothio-diurethane.

Ethylene monothio-diurethane was heated under reflux with concentrated hydrochloric acid for half an hour when a solution was obtained remaining clear on cooling. A small portion after neutralisation with alkali was found to yield no solid product, so the whole was diluted with water and evaporated to dryness on the water-bath when a crystalline residue was left, very soluble in water, alcohol and acetone; it was therefore washed with ether (in which it is almost insoluble) to remove any tarry matter. In order to isolate the free base it was dissolved in the least amount of water and then sodium acetate added when a solid was obtained.[1] This on being crystallised from dilute alcohol melted at 193°, identical with compound (XVII).

[1] The free base can also be isolated by boiling with alcohol.

Action of Potassium Hydroxide on Ethylene monothio-diurethane.

Ethylene monothio-diurethane was mixed with caustic potash (20 per cent.) and allowed to stand at the ordinary temperature for twenty-four hours. It was then heated at 60–70° under reflux for two hours when a clear solution was obtained. The solution was then allowed to cool and acidified with dilute hydrochloric acid when hydrogen sulphide was evolved and a small quantity of sulphur separated. The solution was filtered from sulphur and the filtrate evaporated to dryness. The solid residue was extracted with absolute alcohol and from the alcoholic solution on cooling ethylene thiourea was obtained on dilution with water.

Semicarbazide monothiocarboxylic ester (XVIII).—Semicarbazide hydrochloride (4·5 g.) dissolved in the least quantity of water was treated with anhydrous sodium carbonate (2 g.) to liberate the free base. The solution was diluted with alcohol and added to the required quantity of the ester. Reaction began in the cold with development of some heat and liberation of hydrogen sulphide. The mixture was allowed to stand for about two hours at the ordinary temperature and then heated under reflux for half an hour; needle-shaped crystals were found to have been formed overnight, which after filtration were crystallised from water, m.p. 161°. It is soluble in dilute acid and dilute alkali. On being boiled with strong hydrochloric acid it is decomposed into alcohol, carbon oxysulphide and semicarbazide hydrochloride (Found: N, 26·20. $C_4H_9O_2N_3S$ requires N, 25·76 per cent.).

Thiosemicarbazide carboxylic ester (XIX).—An alcoholic solution of the ester (2 g.) and thiosemicarbazide (2 g.) were heated for an hour under reflux when a clear solution was obtained. Hydrogen sulphide was evolved during the reaction. On allowing the solution to cool a solid separated which after filtration was crystallised from water, m.p. 1551–56°. The substance is soluble in dilute alkali but insoluble in dilute acid (Found: N, 25·86. $C_4H_9O_2N_3S$ requires N, 25·76 per cent.). The filtrate from (XIX) was evaporated to dryness and a second crop of the substance obtained.

Action of Hydrochloric Acid on Thiosemicarbazide carboxylic ester: Formation of (XXIII).—Thiosemicarbazide carboxylic ester was heated with strong hydrochloric acid for half an hour when it passed into solution; this was diluted with water and evaporated to dryness. The white crystalline solid (XXIII) thus obtained was recrystallised from water. It shrinks at 179° and melts at 189°. The substance is chlorine-free and liberates carbon dioxide from sodium bicarbonate. Though it contains sulphur it does not give a mercaptide or a disulphide with mercuric chloride and iodine respectively. It is not desulphurised

when boiled with freshly prepared yellow mercuric oxide, so the sulphur atom is present in the ring. It gives a benzylidene derivative which melts at 158–159° (Found: N, 24·89. $C_3H_3O_3N_3S$ requires N, 25·40 per cent.).

4-*Phenylthiosemicarbazide carboxylic ester* (XX and XXI).—To 2 gms. of the ester, 4-phenylthiosemicarbazide (3·5 gms.) was added. The reaction took place with evolution of hydrogen sulphide and rise of temperature. After allowing the mixture to stand for two hours it was diluted with alcohol and then heated under reflux for about half an hour. The solid was filtered and crystallised from alcohol; m.p. 149–150°. It is soluble in dilute alkali and can be precipitated by dilute acid (Found: N, 17·23; S, 12·85. $C_{10}H_{13}O_2N_3S$ requires N, 17·57; S, 13·38 per cent.).

4-para-*Tolylthiosemicarbazide carboxylic ester* (XXI).—In a manner similar to the preceding reaction *p*-tolylthiosemicarbazide was condensed with the ester. The product was crystallised from a mixture of pyridine and water; m.p. 183–184°. It is insoluble in dilute acid but soluble in alkali (Found: N, 16·74. $C_{11}H_{15}O_2N_3S$ requires N, 16·53 per cent.).

β-*Naphthylthiosemicarbazide carboxylic ester* (XXII) was obtained in a similar manner from β-naphthylthiosemicarbazide (4 g.) and the ester (2 g.). The product was crystallised from pyridine; m. p. 287–288°. It is soluble in alkali but can be precipitated with acid (Found: N, 14·71. $C_{14}H_{15}O_2N_3S$ requires N, 14·52 per cent.).

Benzidine monothiodicarboxylic ester (XXIV).—An alcoholic solution of the ester (2 g.) was added to benzidine (2 g.) under ice cooling. The reaction took place with rise of temperature and liberation of hydrogen sulphide. The reaction mixture was allowed to stand at the ordinary temperature for two to three hours and then heated under reflux for half an hour, the solid product obtained on cooling being filtered and crystallised from acetic acid. The filtrate on evaporation gave a second crop, m.p. 211–212°. It is soluble in dilute alkali but insoluble in dilute acid (Found: N, 8·41. $C_{18}H_{20}O_3N_2S$ requires N, 8·13 per cent.).

Tolidine monothiodicarboxylic ester (XXV) was obtained and purified as in the previous case; m.p. 125–26°. The substance is soluble in alkali but insoluble in acid (Found: N, 7·61. $C_{20}H_{24}O_3N_2S$ requires N, 7·52 per cent.).

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II.—RING CLOSURE OF HYDRAZO-MONOTHIODICARBONAMIDES WITH ACETIC ANHYDRIDE: FORMATION OF IMINOTHIOBIAZOLONES AND IMINOTHIOLTRIAZOLES.[1]

By Praphulla Chandra Guha and Tarani Kanta Chakraborty.

The ring-closing action of acetic anhydride upon hydrazo-dithiodicarbonamide and its substituted derivatives has been studied by one of us (Guha, *J. Amer. Chem. Soc.*, 1923, **45**, 1036) where it has been shown that the reaction takes place with elimination of sulphuretted hydrogen yielding 2 : 5-diamino-1 : 3 : 4-thiodiazoles. In the present paper, the ring-closure of the following substituted hydrazodicarbonamides with acetic anhydride has been studied of which I–VII are substituted in the thiocarbonamide group, VIII and X are substituted in the carbonamide group, IX is substituted both ways and XI is an unsubstituted dicarbonamide.

$$CH_3{\cdot}NH{\cdot}CS{-}NH{-}NH{-}CO{-}NH_2 \quad (I)$$

$$C_3H_5{\cdot}NH{-}CS{-}NH{-}NH{-}CO{-}NH_2 \quad (II)$$

$$C_6H_5{\cdot}NH{-}CS{-}NH{-}NH{-}CO{-}NH_2 \quad (III)$$

$$o\text{-}CH_3{\cdot}C_6H_4{-}NH{-}CS{-}NH{-}NH{-}CO{-}NH_2 \quad (IV)$$

$$p\text{-}CH_3{\cdot}C_6H_4{-}NH{-}CS{-}NH{-}NH{-}CO{-}NH_2 \quad (V)$$

$$(CH_3)_2C_6H_3{\cdot}NH{-}CS{-}NH{-}NH{-}CO{-}NH_2 \quad (VI)$$

$$\beta\text{-}C_{10}H_7{-}NH{-}CS{-}NH{-}NH{-}CO{-}NH_2 \quad (VII)$$

$$NH_2{-}CS{-}NH{-}NH{-}CO{-}NHPh \quad (VIII)$$

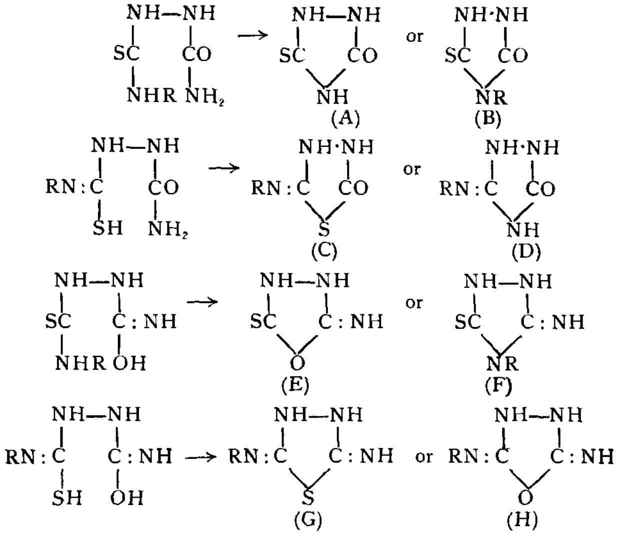
$$NHPh{-}CS{-}NH{-}NH{-}CO{-}NHPh \quad (IX)$$

$$NH_2{-}CS{-}NH{-}NH{-}CO{-}NH{\cdot}C_{10}H_7(a) \quad (X)$$

$$NH_2{-}CO{-}NH{-}NH{-}CO{-}NH_2 \quad (XI)$$

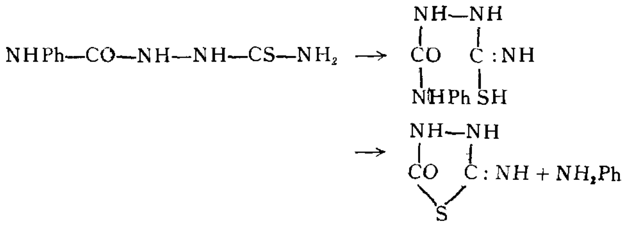
[1] Reprinted from the *Journal of the Indian Chemical Society*, 1929, 6, 99.

Monosubstituted hydrazo-monothiodicarbonamides of the type NHR—CS—NH—NH—CO—NH_2 might react in the following four different ways to yield one or more cycloids of the type A to H.

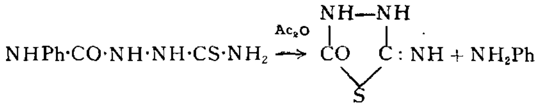


But the compounds actually isolated correspond to the general formula $C_2H_2RON_3S$ and so cannot possess the structure represented by formulae A, D, E, F, G or H, F and G being free from oxygen, D and H being free from sulphur, A and E being free from the group R. So the formula must be B or C, and as the compounds are not mercaptans only C meets all the points. The acetyl derivatives of the substituted iminothiobiazolones are invariably obtained from the hydrazides I–VI which on deacetylation give the free R-iminothiobiazolones. Besides the formation of the acetyl-R-iminothiobiazolones, indication is given as to the formation of other products and the actual isolation of more than one has been possible with the hydrazides II, III and V. It is worth mentioning that one product from the hydrazide V happens to be an iminothioltriazole of type F. The hydrazide VII is completely decomposed with the regeneration of β-naphthyl *iso*thiocyanate almost in a quantitative yield.

The hydrazide VIII under mild treatment gives mono- and diacetyl derivatives of iminothiobiazolone. The ring closure is evidently effected with elimination of aniline, thus :—



When boiled with acetic anhydride over a free flame, the hydrazide is decomposed yielding diphenylurea, acetanilide, a trace of mono-acetyliminothiobiazolone and two compounds melting at 92–93° and 115° respectively. The formation of acetyliminothiobiazolone, diphenylurea and acetanilide can be explained thus :—

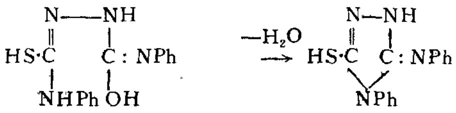


$$NHPh{\cdot}CO{\cdot}NH{\cdot}NH{\cdot}CS{\cdot}NH_2 \xrightarrow{Ac_2O} NPh{:}CO + NH_2{\cdot}NH{\cdot}CS{\cdot}NH_2$$ [1]

$$2\ NPh{:}CO + H_2O \longrightarrow CO(NHPh)_2 + CO_2.$$

The formation of unsubstituted iminothiobiazolone in the present instance and of substituted iminothiobiazolones with the hydrazides containing substituted thiocarbonamide groupings is significant.

The behaviour of the *sym*-disubstituted hydrazide, NHPh·CS·NH·NH·CO·NHPh appears to be peculiar, as instead of yielding the expected iminothiobiazolone derivatives, it gives 1-*N*-phenyl-2-phenylimino-5-thiol-dihydro- 1 : 3 : 4-triazole, thus :—



[1] The analytical values of the compound, m.p. 92–93°, agree fairly well with those of diacetyl thiosemicarbazide (Calc. N, 24·28 ; S, 18·5. Found : N, 24·70 ; S, 18·67 per cent.).

When boiled with acetic anhydride, the hydrazide yields diphenylurea as the main product. The hydrazide IX, like VII, is easily decomposed and the α-napthyl *iso*cyanate so formed reacts readily with water to give di-α-napthylcarbamide.

Hydrazodicarbonamide (XI) is unaffected even when boiled with acetic anhydride for several days, but when the mixture is heated in a sealed tube at 200° is decomposed into various products amongst which carbon dioxide and *sym*-diacetylhydrazine have been identified.

EXPERIMENTAL.

Hydrazodicarbo-monothiomethylamide (I).

Semicarbazide hydrochloride (5·6 g.) was dissolved in the least quantity of water and sodium carbonate (2·7 g.) added. Molecular proportion of methyl mustard oil dissolved in the least quantity of alcohol was added and the mixture was warmed. Within a minute or two the hydrazide separated, and was crystallised from alcohol, melting at 212°; the yield was quantitative (Found: N, 37·62. $C_3H_8ON_4S$ requires N, 37·84 per cent.).

2-*Methylimino*-4-*acetyl*-5-*keto*-2 : 3 : 4 : 5-*tetrahydro*-1 : 3 : 4-*thiodiazole*.

The above hydrazide (5 g.) was heated with a slight excess of acetic anhydride, the hot clear solution poured into 100–120 c. c. of cold water and the aqueous solution boiled for some time when an oily product separated which, however, solidified after 24 hours. It was filtered, washed with ether and crystallised from alcohol. It is very slightly soluble in water and in acid; alkali decomposes it producing a faint smell of *iso*cyanide; m.p. 197°, yield 1·5 gms. (Found: N, 24·02. $C_5H_7O_2N_3S$ requires N, 24·3 per cent.).

Hydrolysis to the thiodiazole.—Two grams of the above acetate were boiled with strong hydrochloric acid for about three minutes, becoming dissolved and on evaporation yielding the free basic thiodiazole. It was filtered and further purified by crystallisation from water, m.p. 232° (Found: N, 31·57. $C_3H_5N_3OS$ requires N, 32·06 per cent.).

Hydrazomonothioallyldicarbonamide (II).

Semicarbazide hydrochloride (5 g.) was dissolved in water, treated with anhydrous sodium carbonate (2·4 g.) and boiled on a water-bath with an alcoholic solution of allyl mustard oil (4·5 g.) for five minutes

when crystals of the hydrazide began to separate as shining plates. The reaction was complete within ten minutes. The hydrazide was purified by crystallisation from alcohol; m.p. 202° (Found; N, 32·52. $C_5H_{10}ON_4S$ requires N, 32·18 per cent.).

2-Allylimino-3-acetyl-5-keto-2 : 3 : 4 : 5-tetrahydro-1 : 3 : 4-thiodiazole.

A few grams of the hydrazide were just covered with acetic anhydride and heated under reflux over a small free flame until a clear solution was obtained. On pouring the solution into water, an oil separated which solidified on keeping under water for a few days. The yield of the solid was very small and the presence of a considerable quantity of mustard oil was detected, showing that the major portion of the hydrazide had undergone decomposition. The solid was washed with ether and crystallised from alcohol; m.p. 137°. The yield was so small that no further investigation of the substance was possible.

The experiment was repeated by heating the hydrazide with acetic anhydride on a water-bath instead of over a free flame; the resulting oil soon solidified yielding a much greater quantity of the substance. It was washed with ether, and on attempting crystallisation from rectified spirit was found to be a mixture of two substances. The solid was dissolved by heating with 60 per cent. alcohol and filtered while hot. The filtrate yielded on cooling a crystalline substance, representing about 90 per cent. It was again crystallised from rectified spirit; m.p. 171° (Found: N, 21·33; S, 15·65. The monoacetyl compound, $C_7H_9O_2N_3S$ requires N, 21·11; S, 16·08 per cent.).

The substance dissolves in cold dilute alkali but is not acted on by oxidising agents such as iodine solution or hydrogen peroxide. It was deacetylated by heating with moderately strong hydrochloric acid when it dissolved, yielding on concentration crystals of the free thiodiazole; m.p. 210° (Found: N, 26·83. $C_5H_7ON_3S$ requires N, 26·75 per cent.).

The mother-liquor was concentrated on a water-bath to very small bulk, filtered and allowed to cool when a white crystalline substance separated, m.p. 269°, and sparingly soluble in cold alkali, but more in hot, giving the smell of *iso*cyanide. The yield was only 0·05 g. and so it could not be analysed.

Hydrazomonothiophenyldicarbonamide (III) *and acetic anhydride. Formation of 2-Phenylimino-3-acetyl-5-keto-2 : 3 : 4 : 5-tetrahydro-1 : 3 : 4-thiodiazole and 2-Phenylimino-3 : 4-diacetyl-5-keto-2 : 3 : 4 : 5-tetrahydro-1 : 3 : 4-thiodiazole.*

The hydrazide, $NH_2\cdot CO\cdot NH\cdot NH\cdot CS\cdot NHPh$, prepared according to the method of Arndt, Milde and Tschenscher (*Ber.*, 1922, **55**, 349)

was heated over a small flame with acetic anhydride sufficient to cover it, in presence of a little well powdered fused sodium acetate. The clear solution thus obtained was poured into water when an oil with a strong smell of phenyl mustard oil separated and solidified on keeping under water for a few days. The yellow solid was washed successively with rectified spirit and ether, dissolved in boiling dilute alcohol, the solution filtered and the filtrate allowed to stand for some time while a good quantity of crystals separated. They were filtered, and the mother-liquor on concentration gave another crop of crystals of different shape and appearance from the former. The two portions were separately collected and gave different and fairly sharp melting points (210–211°) and (171–172°) respectively. After recrystallisation from dilute alcohol, the first portion melted at 213° and was the diacetyl compound (Found: N, 15·47. $C_{12}H_{11}O_3N_3S$ requires N, 15 per cent.). The second portion melted at 173° and was the monoacetyl compound (Found: N, 18·01. $C_{10}H_9O_2N_3S$ requires N, 17·95 per cent.).

2-*Phenylimino*-5-*ketotetrahydro*-1:3:4-*thiodiazole.*

The above acetyl compounds were separately hydrolysed with strong hydrochloric acid. A crystalline white precipitate was slowly produced from each solution on adding water and was crystallised from dilute alcohol, melting at 206°. It slowly dissolves in cold dilute alkali with the development of a pale green colour and the disagreeable smell of *iso*cyanide (Found: N, 21·98. $C_8H_7ON_3S$ requires N, 21·76 per cent.).

The hydrazide was also heated with acetic anhydride in presence of a small quantity of fused sodium acetate on a water-bath instead of over a free flame, with greatly increased yield. Only a small fraction of the hydrazide was acted on during a period of two hours. Only the monoacetyl compound was obtained in this way.

Hydrazomonothio-ortho-*tolyldicarbonamide* (IV).

Semicarbazide hydrochloride (5 g.) dissolved in the least quantity of water was treated with anhydrous sodium carbonate (2·4 g.) and heated under reflux with *o*-tolyl mustard oil (6·6 g.) for twenty minutes while the hydrazide separated as a white solid. It was crystallised from alcohol; m.p. 201° with decomposition (Found: N, 25 12. $C_9HO_{12}N_4S$ requires N, 25·00 per cent.).

2-o-*Tolylimino*-3-*acetyl*-5-*keto*-2:3:4:5-*tetrahydro*-1 : 3 : 4-*thiodiazole.*

The above hydrazide was covered with acetic anhydride in presence of a little powdered fused sodium acetate and carefully heated

with a small flame until the reaction commenced, yielding a clear solution which gave an oily product on being poured into water. After 24 hours' standing the solidified oil was washed well with alcohol and ether and crystallised from rectified spirit; m.p. 183° (Found: N, 17·10. $C_{11}H_{11}O_2N_3S$ requires N, 16·87 per cent.).

2-o-Tolylimino-5-ketotetrahydro- 1 : 3 : 4-thiodiazole.

The above acetyl compound was boiled under reflux with fuming hydrochloric acid but was not dissolved. The solid product was washed with water, dried and crystallised from a large quantity of dilute alcohol; m.p. 210° (Found: N, 20·44. $C_9H_9ON_3S$ requires N, 20·29 per cent.).

*Hydrazomonothio-*p*-tolyldicarbonamide* (V).

Semicarbazide hydrochloride (5 g.) dissolved in the least quantity of water was treated with anhydrous sodium carbonate (2·4 g.) and boiled under reflux with *p*-tolyl mustard oil (6·6 g.). After 15 minutes, crystals of the hydrazide began to separate and the reaction came to an end after about half an hour. It was crystallised from alcohol; m.p. 192° (Found: N, 25·32. $C_9H_{12}ON_4S$ requires N, 25·00 per cent.).

*Hydrazomonothio-*p*-tolyldicarbonamide and acetic anhydride. Formation of 2-*p*-Tolylimino-5-keto-2:3:4:5-tetrahydro-1:3:4-thiodiazole and* p*-Tolyl-2-imino-5-thiol-3-acetyl-2:3-dihydro-1:3:4-triazole.*

The clear solution obtained within a few minutes' heating was poured into cold water and allowed to stand overnight. Next morning the oil was found to have partially solidified with a layer of crystals deposited over it. The white solid that came from the aqueous solution was carefully collected and crystallised from rectified spirit; m.p. 247°. It was 2-*o*-tolylimino-5-keto-2:3:4:5-tetrahydro-1:3:4-thiodiazole, insoluble in alkali and remaining unchanged on being boiled with fuming hydrochloric acid pointing to the fact that there was no acetyl group present (Found: N, 20·61. $C_9H_9ON_3S$ requires N, 20·29 per cent.). The solidified oil was washed carefully with ether and crystallised twice from rectified spirit; m.p. 154°. It readily dissolves in alkali from which acid precipitates it. It is acted on by ferric chloride, hydrogen peroxide and iodine solution. It is 2-*o*-tolylimino-3-acetyl-5-thiol-2:3-dihydro-1:3:4-triazole (Found: N, 21·97; S, 12·83. $C_{11}H_{12}ON_4S$ requires N, 22·58; S, 12·90 per cent.). As the quantity was very small it could not be deacetylated with hydrochloric acid.

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Hydrazomonothio-xylyldicarbonamide (VI).

Semicarbazide hydrochloride (5 g.) dissolved in a small quantity of water was treated with anhydrous sodium carbonate (2·4 g.) and boiled under reflux with xylyl mustard oil (7·2 g.) in about 200 c.c. alcohol. Within half an hour the reaction was complete and the separated solid was crystallised from alcohol, m. p. 200° with decomposition (Found: N, 23·81. $C_{10}H_{14}ON_4S$ requires N, 23·53 per cent.).

2-*Xylylimino*-3-*acetyl*-5-*keto*-2 : 3 : 4 : 5-*tetrahydro*-1 : 3 : 4-*thiodiazole*.—The hydrazide was treated as usual with fused sodium acetate and acetic anhydride and within two minutes a clear solution was obtained which was poured into water. An oil separated smelling strongly of mustard oil and solidified after 24 hours. It was washed with ether and crystallised from rectified spirit, m. p. 218° (Found: N, 16·00. $C_{12}H_{13}O_2N_3S$ requires N, 15·97 per cent.).

Hydrolysis.—The above acetyl compound was boiled for half an hour under reflux with fuming hydrochloric acid, and the product crystallised from rectified spirit; m.p. 232°. The substance slowly dissolved in cold dilute alkali with a pinkish colour which disappears on boiling but reappears on cooling (Found: N, 18·68. $C_{10}H_{11}ON_3S$ requires N, 19·00 per cent.).

Hydrazo-monothio-β-naphthyldicarbonamide (VII).

β-Naphthyl mustard oil (5 g.) was dissolved in alcohol by warming and to this was added an aqueous solution of semicarbazide hydrochloride (3 g.) treated previously with anhydrous sodium carbonate (1·4 g.). The mixture was then gently boiled under reflux for about 10 minutes and the separated hydrazide after washing with alcohol was crystallised from a large volume of the same solvent; m.p. 210° (decomp.) (Found: N, 21·10. $C_{12}H_{12}ON_4S$ requires N, 21·54 per cent.).

The hydrazide on being heated with acetic anhydride (though extremely carefully) decomposed completely. Among the decomposition products almost the calculated quantity of β-naphthyl mustard oil was isolated and identified.

Hydrazomonothio-phenyldicarbonamide (VIII) *and acetic anhydride. Formation of* 2-*Imino*-3-*acetyl*-5-*keto*-2 : 3 : 4 : 5-*tetrahydro*-1 : 3 : 4-*thiodiazole and* 2-*Imino*-3 : 4-*diacetyl*-5-*keto*-2 : 3 : 4 : 5-*tetrahydro*-1 : 3 : 4-*thiodiazole*.

Hydrazomonothiophenyldicarbonamide, NHPh·CO·NH·NH·CS·NH$_2$, prepared from phenyl *iso*cyanate and

thiosemicarbazide according to Freund and Schander (*Ber.*, 1896, **29**, 2510) was treated with acetic anhydride in the usual way. The solid obtained from the oil after 24 hours was crystallised from rectified spirit, m.p. 275° (Found: N, 26·52, 26·72; S, 19·90. The monoacetyl compound, $C_4H_5O_2N_3S$ requires N, 26·42; S, 20·13 per cent.).

The solution after the separation of the solidified oil was concentrated to small bulk, and steam passed through it until the solution was almost free from acetic acid. The volume was again considerably reduced by evaporating on a water-bath and crystals came out on cooling; these were recrystallised from a mixture of water and alcohol, m.p. 160° (Found: N, 21·04. The diacetyl compound, $C_9H_7O_3N_3S$ requires N, 20·90 per cent.). The free thiodiazole derivatives could not be prepared by deacetylation with hydrochloric acid for want of sufficient quantity of the acetyl derivatives.

In another experiment the hydrazide was boiled with excess of acetic anhydride for about 10 minutes and the clear solution found to smell of phenyl *iso*cyanate. The reaction mixture on being poured into water gave an oil which solidified on standing, and from which diphenylurea and acetanilide were isolated. The aqueous solution was evaporated to a syrup and kept in a desiccator containing potassium hydroxide under vacuum for two to three days when it was found not to solidify. It was then dissolved in water, steamed until free from acetic acid and evaporated almost to dryness. On cooling a solid separated which was found to be partially soluble in sodium hydroxide solution. This alkaline solution after acidification was evaporated to dryness, extracted with absolute alcohol and the alcoholic extract gave on concentration and cooling a crystalline mass, m.p. 92–93° (Found: N, 24·7; S. 18·67 per cent.).

The alkali insoluble solid on repeated fractional crystallisation from spirit yielded two compounds melting respectively at 115° and 296°. The former on analysis gave C, 42·61; H, 5·01, and the latter gave S, 19·2; N, 26·72 ($C_4H_5O_2N_3S$ requires S, 20·13; N, 26·42) and is evidently identical with acetyl iminothiobiazolone of Guha (*J. Amer. Chem. Soc.*, 1923, **55**, 1042).

Hydrazomonothio-sym-*diphenyldicarbonamide* (IX) *and acetic anhydride. Formation of* 1-*Phenyl*-2-*phenylimino*-5-*thiol*-2 : 3-*dihydro*-1 : 3 : 4-*triazole.*

Hydrazomonothio-*sym*-diphenyldicarbonamide, was prepared from 4-phenylthiosemicarbazide and phenyl *iso*cyanate. The solidified oil

obtained after the usual treatment was washed with ether and crystallised twice from alcohol. It melted at 208°. The substance was soluble in alkali and unchanged by boiling with strong hydrochloric acid, showing that no acetyl group was present (Found: N, 21·06. $C_{14}H_{12}N_4S$ requires N, 20·90 per cent.). On heating with excess of acetic anhydride for about ten minutes the main product was diphenylurea, m.p. 236° mixed with a small quantity of the alkali soluble substance, m.p. 208°.

Hydrazomonothio-α-naphthyldicarbonamide (X).

Thiosemicarbazide (4 g.) dissolved in the least quantity of boiling water was treated with a few drops of acetic acid; to this was gradually added α-naphthyl *iso*cyanate (7·4 g.) in about 200 c.c. of absolute alcohol. A vigorous reaction took place instantaneously, the hydrazide came out as a white solid and was crystallised from rectified spirit; m.p. 213° (Found: N, 21·68. $C_{12}H_{12}ON_4S$ requires N, 21·54 per cent.).

Hydrazomonothio-α-naphthyldicarbonamide and acetic anhydride.

The hydrazide was covered with acetic anhydride in presence of a little powdered fused sodium acetate and gently heated to boiling for about five minutes; the turbid solution was poured into water through a filter. The separated oil was stirred vigorously for ten minutes with hot water and within an hour or so the oil solidified, being freed from tar by washing successively with acetone, ether and benzene. It was then crystallised twice from boiling pyridine; m.p. 293°. The compound contains no sulphur, is insoluble in acid and alkali and in all solvents excepting boiling pyridine (Found: N, 9·18 per cent.). It is therefore *sym*-α : α′-dinaphthyl urea (Cal. N, 8·97 per cent.).

Hydrazodicarbonamide (XI) *and acetic anhydride: Formation of* sym-*Diacetylhydrazine.*

Hydrazodicarbonamide (10 g.) prepared according to the method of Thiele (*Annalen*, 1892, **271**, 127) was heated with 30–40 c.c. of pure acetic anhydride in a sealed tube at 200° in an oil-bath for two hours when all dissolved. On opening the tube carbon dioxide escaped under great pressure. The solution was filtered from a small quantity of hydrazodicarbonamide and steam passed for four hours to remove acetic acid. The solution was then evaporated to a brown syrup on the water-bath, dissolved in acetone and boiled under reflux for thirty minutes with animal charcoal. After filtration and concentration,

excess of ether was added to the cold syrup under vigorous stirring when a beautiful crystalline mass separated. It was purified by precipitating again from an acetone solution and finally crystallised from benzene; m.p. 139° (yield, 4 gms.). It was proved to be *sym*-diacetylhydrazine (*m.p.* 138°) and on being heated with strong hydrochloric acid gave hydrazine dihydrochloride, m.p. 200°.

Our thanks are due to Mr. Sreekumaran Unni Nair for some preliminary work in connection with this paper.

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STUDIES IN ENZYME ACTION. PART III. AMYLASE FROM CUMBU (*Pennisetum typhoideum*).

By D. Narayanamurti, C. V. Ramaswami Ayyar and Roland V. Norris.

Effront (*Compt. rend.*, 1922, **174**, 18) has shown that the ratio $\frac{\text{saccharification power}}{\text{liquefaction power}}$ can vary from 0·1 to 2,400 depending on the source from which the diastase has been prepared. The experiments of Windisch, Dietrich and Beyer (*Woch. Brau.*, 1923, Nos. 40, 49, 55, 61, 67) also indicate that the ratio is not constant for all malts, thus suggesting that diastase consists of two components and that these occur in different proportions in the various grains. The experiments of Narayanamurti and Norris (*J. Ind. Inst. Sc.*, 1928, **11 A**, 134) produced strong evidence in favour of the two enzyme theory. A detailed investigation of diastase from different sources has therefore been undertaken, and this part is concerned with the optimum P_H of the enzyme prepared from malted cumbu, under varying conditions of time, temperature and age.

EXPERIMENTAL.

The enzyme was extracted from about 200 gms. of malt with 600 c.c. of distilled water in presence of toluene, dialysing the filtered extract in collodion bags in flowing distilled water for a week. Hydrolysis took place in an electrically controlled thermostat and the sugar was estimated according to the iodometric method of Schaffer and Hartman (*J. Biol. Chem.*, 1920–1921, **45**, 365).

Optimum P_H and period of hydrolysis.—Four periods 15, 30, 60 and 140 minutes have been studied. The results are given in Table I and Figure I.

TABLE I.

The reaction mixture contained 5 c.c. starch solution (0·5 per cent.), 5 c.c. enzyme solution, 10 c.c. Walpole's acetate buffer and a few crystals of thymol as antiseptic. The temperature was 37°.

P_H	Maltose in c.c. thiosulphate			
	15 mins.	30 mins.	60 mins.	140 mins.
6·5	1·8	2·9	6·7	11·0
6·2	2·4	5·0	8·9	12·6
6·0	2·7	5·5	8·7	12·5
5·9	2·9	...	8·5	11·9
5·6	3·3	6·0	9·6	13·2
5·4	3·5	6·4	9·9	13·2
5·2	3·6	6·4	...	13·5
5·1	3·9	6·5	...	13·8
5·0	...	6·6	10·2	13·8
4·8	3·7	6·7	...	14·2
4·6	3·6	6·2	...	14·1
4·5	3·5	6·1	10·0	13·6
4·3	3·4	6·0	9·4	...
4·0	...	5·0	8·7	12·5
3·7	1·1	3·8	6·9	10·9
3·3	0·7	1·2	4·2	6·0

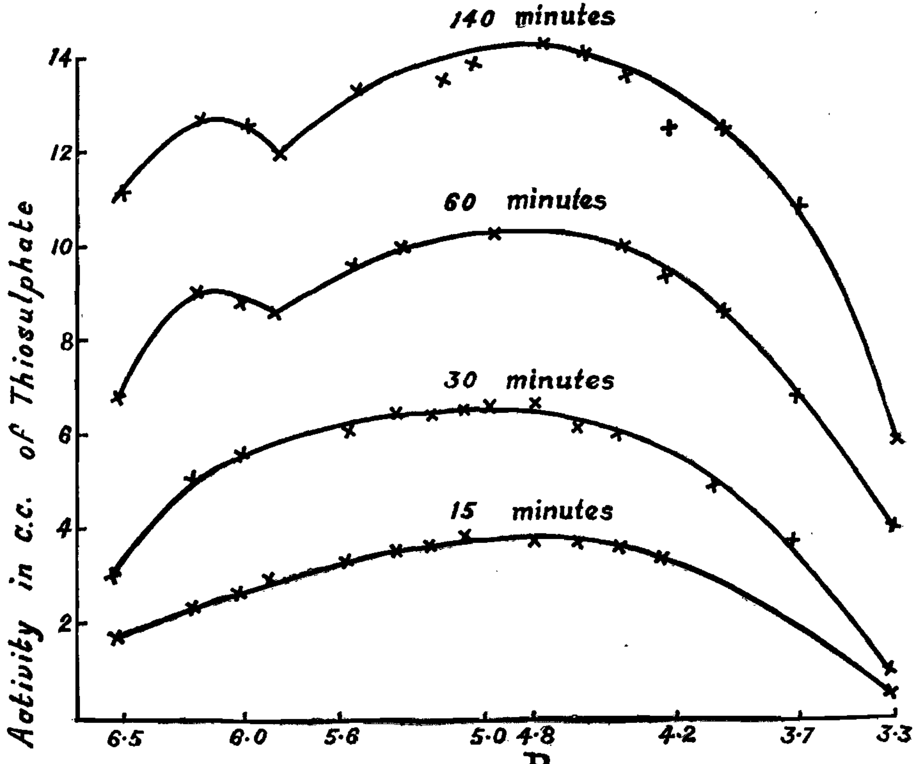
It is evident that the optimum P_H for 15 minutes hydrolysis lies at about 5·1 and is slightly diverted to the acid side for longer periods. The occurrence of maltase in malt has recently been confirmed by Leibowitz (*Z. Physiol. Chem.*, 1925, **149**, 184; 1926, **154**, 64) who showed that maltase acts best at P_H 4·0–4·6. This suggested that the slight deviation observed is due to the occurrence of maltase in cumbu malt, and on testing the enzyme solution with maltose the presence of maltase was confirmed; this was found to act best at a P_H of 4·6 (see Table II).

TABLE II.

The reaction mixture contained 5 c.c. maltose (0·2 per cent.), 5 c.c. enzyme, 10 c.c. buffer and a few crystals of thymol as antiseptic. The temperature was 37°.

P_H ...	6·2	5·9	5·2	5·1	4·8	4·6	4·5	4·3	4·0
Glucose (c.c. thiosulphate) ...	0·2	0·3	1·2	1·5	1·6	1·7	1·3	1·2	0·8

For periods over 30 minutes there are two optima, at about 4·8 and 6·2. A similar observation has been made with regard to emulsion by Brunswik (*Osterr. botan. Z.*, 1923, No. 1–5, 58), and was explained



140 minutes
60 minutes
30 minutes
15 minutes
Activity in c.c. of Thiosulphate
14
12
10
8
6
4
2
6·5
6·0
5·6
5·0
4·8
4·2
3·7
3·3
PH

Fig. I

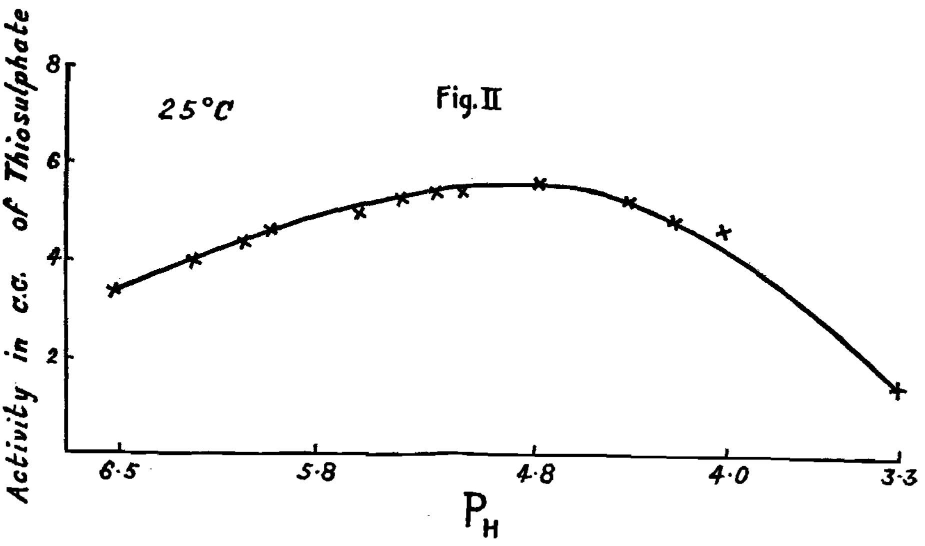


Fig. II

Fig. III

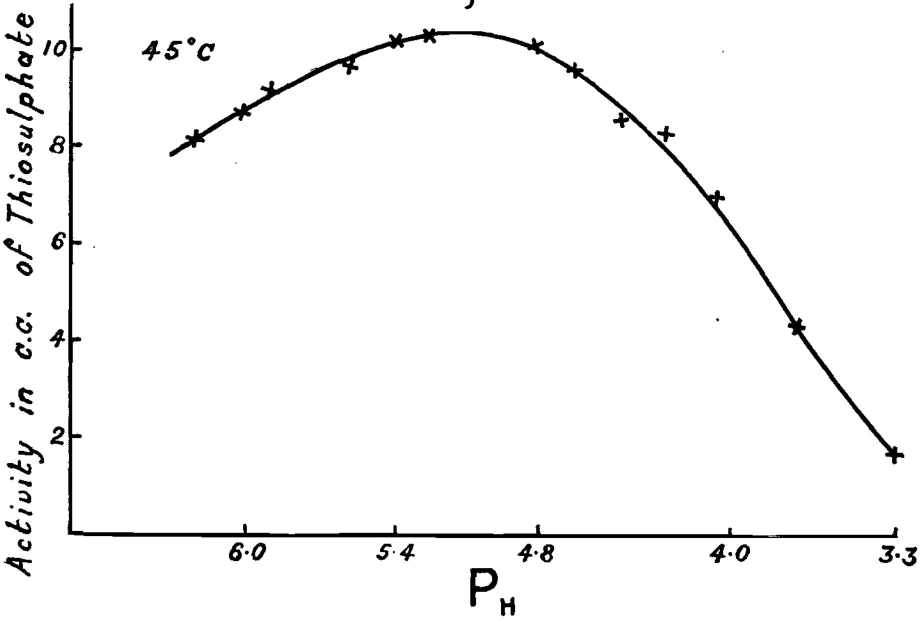
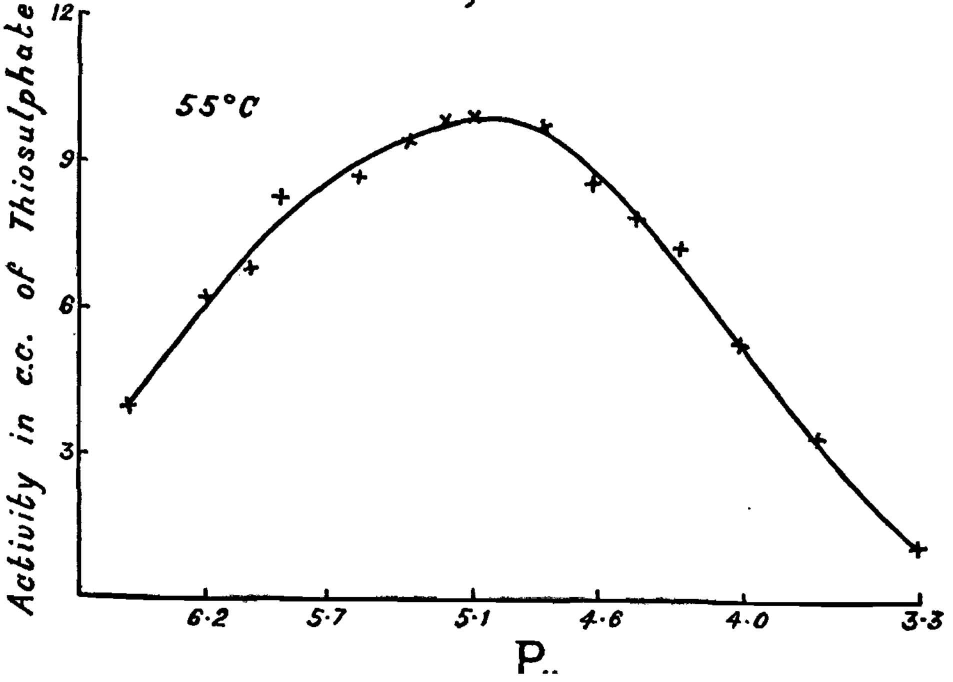


Fig. IV



as due to the presence of amygdalase and prunase in the enzyme preparation. The point is being carefully investigated to see whether in diastase also it is due to two components.

The effect of temperature is recorded in Table III and Figures II, III and IV.

TABLE III.

The reaction mixture contained 5 c. c. starch (0·5 per cent.), 5 c. c. enzyme solution, 10 c. c. Walpole's acetate buffer and a few crystals of thymol as antiseptic. The period of hydrolysis was 30 minutes.

P_H	Maltose in c. c. thiosulphate		
	Temperature		
	25°	45°	55°
6·5	3·4	...	3·9
6·2	4·0	8·2	6·2
6·0	4·0	8·8	6·8
5·9	4·6	9·2	8·3
5·6	5·0	9·7	8·7
5·4	5·4	10·2	9·4
5·2	5·5	10·3	9·9
5·1	5·5	...	9·9
4·8	5·7	10·1	9·7
4·6	...	9·6	9·6
4·5	5·3	8·6	7·9
4·3	5·0	8·4	7·2
4·0	4·8	7·0	5·2
3·7	3·3	4·3	3·3
3·3	1·6	1·7	1·1

With rise in temperature we find a slight deviation to less acid hydrogen-ion concentration. Probably the enzyme is more stable in these hydrogen-ion concentrations. We have to distinguish two effects of temperature on the enzyme, acceleration of action and destruction of

the enzyme. Ernstrom (*Z. Physiol. Chem.*, 1922, **119**, 190; 1923, **141**, 40) and Sjoberg (*Biochem Z.*, 1922, **133**, 298) have shown that with rise in temperature the stability optimum is diverted to less acid hydrogen-ion concentrations, agreeing with the above results. Another point to be mentioned is that the zone of optimum P_H becomes narrowed with rise in temperature. The results for 55° are of special interest, for, according to several investigations the temperature of inactivation lies at about this point.

Effect of age.—Preliminary experiments indicate that the P_H optimum is diverted to less acid solutions. Experiments are in progress to follow the changes taking place with age by measuring the conductivity, viscosity, surface tension and other physical constants of the enzyme solution. We are also studying the effect of the nature of the buffer, salt-content and other factors.

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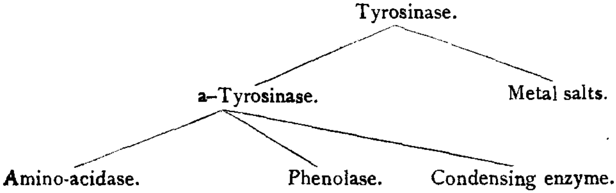
STUDIES IN ENZYME ACTION. PART IV.

Tyrosinase I.

By D. Narayanamurti and C. V. Ramaswami Ayyar.

The enzyme tyrosinase distributed among plants and animals was discovered by Bourquelot and Bertrand (*J. Pharm. Chim.*, 1896, **3**, 177; *Bull. Soc. Mycol.*, 1896, **12**, 17) and has engaged the attention of numerous biochemists; it is able to oxidise tyrosine in presence of atmospheric oxygen. That other oxidising enzymes have no action on tyrosine was first demonstrated by Bertrand who showed that the oxidases, phenolase and tyrosinase could be separated from one another. Gessard noticed its presence in *B. Pyocyaneus* and extracted the enzyme from fungi with glycerine. Russula tyrosinase was used by Harley (*J. Pharm. Chim.*, 1899, **9**, 225 & 424; 1900, **11**, 172) to test for tyrosine in digestion mixtures. Gonnerman (*Chem. Ztg.*, 1916, **40**, 127) isolated active preparations from the potato. Consequently the enzyme most studied has been prepared either from Russula or the potato.

Gessard (*Compt. rend.*, 1900, **130**, 1327) found that the red oxidation product formed by the action of tyrosinase on tyrosine is quickly transformed into melanin by salts of alkaline earth metals and of heavy metals. Though it is quite probable that salts act by coagulating the colloidal melanin formed, it is claimed by Haehn (*Biochem. Z.*, 1920, **105**, 169; *Ber.*, 1919, **52**, 2029; *Fermentforschung*, 1921, **4**, 301) that the enzyme is quite inactive in absence of salts, and he ascribes to these the function of a co-ferment. He believes that the enzyme is a mixture of components which can be represented thus:—



Similar views have been held by Bach, Folpmers (*Biochem. Z.*, 1016, **78**, 180), Onslow (*Biochem. J.*, 1923, **17**, 216) and others.

Raper and collaborators (*Biochem. J.*, 1923, **17**, 454; 1925, **19**, 84, 92; 1926) have elucidated the chemical transformations involved; they recognised three distinct stages in the formation of melanin. In the first stage tyrosinase produces a red substance from tyrosine, only in the presence of oxygen. 'The red substance becomes spontaneously colourless and is then oxidised to melanin. These last two processes take place in the absence of tyrosinase, but may be accelerated by it or other oxidases found in the potato juice.' They have also shown that there are no grounds for assuming that deamination occurs when tyrosinase acts on tyrosine.

During an investigation on the proteins of *Dolichos lablab* it was observed that aqueous extracts of the seed darken on standing in air and this was traced to the enzyme tyrosinase. A survey of the literature showed that, apart from the work of Raper, which is mainly concerned with the chemical mechanism, no systematic and quantitative study of the enzyme has been made. Much of the previous work has been concerned with colour reactions and this might lead to erroneous conclusions, as seen for example in the section on the action of hydrogen peroxide. Moreover, the enzyme prepared from *Dolichos lablab* differs in several respects from the one obtained from the potato. A physico-chemical study of the enzyme is described in this paper.

METHODS.

Preparation of the enzyme.—The enzyme was generally prepared by extracting 180 gms. of the finely ground ungerminated seed of *Dolichos lablab* with 600 c.c. of water in presence of toluene for three days at room-temperature. After filtering through paper pulp the extract (containing a large quantity of protein) was dialysed under slight pressure in collodion bags against flowing distilled water for a week, when all the proteins are precipitated and the solution is now centrifuged to remove suspended matter, the resulting brown liquid being clear. The enzyme thus prepared, unlike the one from potato, is very stable in aqueous solution for many weeks at ice-box temperature.

Estimation of activity.—The method of Raper (*Biochem. J.*, 1923, **17**, 454) as modified by Haehn and Stern (*Biochem. Z.*, 1927, **184**, 182) was adopted, the principle being to estimate the balance of tyrosine left by bromination.

Experimental arrangement.—The reactions were conducted in an electrically regulated thermostat in which all solutions were kept initially for half an hour. The reactions took place in long-necked

round bottomed flasks provided with two-holed rubber stoppers. Through one hole passed a long glass tube reaching to the bottom of the flask and acting as the air-inlet, while the other took a very short tube ending flush with the bottom of the rubber stopper. After the enzyme solution, buffer and tyrosine had been mixed in the right proportion, 10 c.c. of toluene to every 100 c.c. of the reaction mixture were added as an antiseptic, to avoid frothing and unnecessary loss due to evaporation. The reaction mixture was now aerated with the help of an electrically driven 'Cenco' blower, the air previously passed through two wash bottles, one containing toluene and the other distilled water saturated with toluene.

EXPERIMENTAL.

Influence of the quantity of meal on extraction.—W. B. Hardy (*J. Physiol.*, 1905, **33**, 251) and Mellanby (*J. Physiol.*, 1905, **33**, 335) have shown that the well-known colloid solubility of globulins in dilute solutions of electrolytes depends on the quantity of globulin taken. The quantity of globulin going into solution for the same volume of peptiser increases with increasing quantity of the solid phase although some of the globulin is left. Their experiments clearly bring out the difference between the solubility of molecularly dispersed substances and colloid solubility. While a molecularly soluble substance has only one solubility at a given temperature and given solvent independent of the quantity of the solid phase, the peptisation of colloids is not similar. The recent work of Buzagh (*Kolloid Z.*, 1927, **211**, 169; 1927, **43**, 215; 1928, **44**, 156), Neunstein, Wo. Ostwald and his students (*Kolloid Z.*, 1927, **41**, 163; 1927, **43**, 249) has clearly shown that this relation is not confined to globulins, but that all colloids, inorganic as well as organic, exhibit the same behaviour. The theoretical aspect has been recently discussed by Wo. Ostwald (*Kolloid Z.*, 1927, **41**, 163; **43**, 249), who has shown that adsorption plays an important part in this process.

That enzymes might exhibit such a behaviour is conceivable and our experiments bring evidence in strong support of this. Varying quantities of the meal were shaken with 200 c. c. of distilled water and 5 c. c. of toluene for three hours in stoppered bottles of the same size and shape, and the extracts along with the solid residues centrifuged to remove all precipitated and suspended matter. Portions (50 c. c.) of each centrifugate were then dialysed under slight pressure in parchment thimbles of the same size in flowing distilled water. In about five days all the proteins had settled and the solutions were again centrifuged, made up to the same volume and their activities compared. The results are given in Table I and Figure I

TABLE I.

Comparison of extract activities.

The reaction mixture contained 10 c. c. acetate buffer at P_H 6·5, 25 c. c. tyrosine solution (0·25 per cent.), 10 c. c. distilled water, 5 c. c. dialysed enzyme extract and 5 c. c. toluene. The temperature was 25° and the duration of experiment, 2 hours for the extract used soon after dialysis (fresh) and 4 hours for the aged one.

Weight of meal in gms.	Activity in c. c. thiosulphate corresponding to tyrosine oxidised in 20 c. c.	
	Fresh	Aged
20	0·8	0·5
40	2·9	2·8
60	4·1	4·1
70	2·4	6·0
80	1·6	4·4
90	1·8	4·5
100	3·1	7·8
120	3·3	8·0

It is evident from the results that the quantity of enzyme-complex extracted is dependent on the quantity of the solid phase. While the investigations of Buzagh with inorganic colloids indicate that the colloid solubility increases at first with increasing quantity of the substance and then falls with further increase of the solid phase we met with two optima, their position being changed with age. This result indicates that perhaps the enzyme is a mixture of two components with different optima, or that an activator is present in the meal; such an activator must be in the colloidal condition, as indicated by our electro-dialysis and ultra-filtration experiments.

The influence of hydrogen-ion concentration.—Search in the literature showed that this factor has not been determined in the case of tyrosinase. Raper (*Biochem. J.*, 1923, **17**, 454) working with potato tyrosinase found that the activity was greater at P_H 7·0 than 6·0, and greater at P_H 8·0 than 7·0. No clear maximum has been defined. The optimum for the enzyme from *Dolichos lablab* was found to be in the neighbourhood of 6·5. It thus differs from the potato enzyme, the activity at P_H 8·0 being considerably reduced. The optimum was

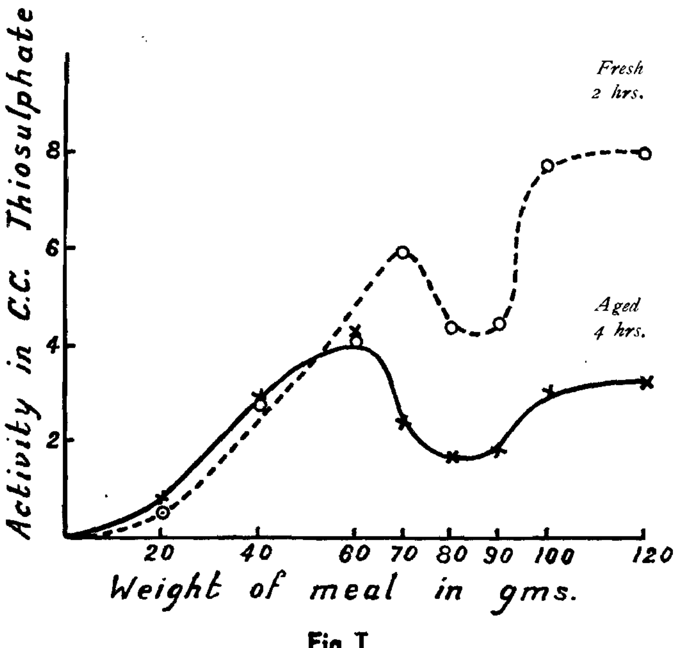

Fresh
2 hrs.
Aged
4 hrs.
Activity in C.C. Thiosulphate
8
6
4
2
20
40
60
70
80
90
100
120
Weight of meal in gms.

Fig. I

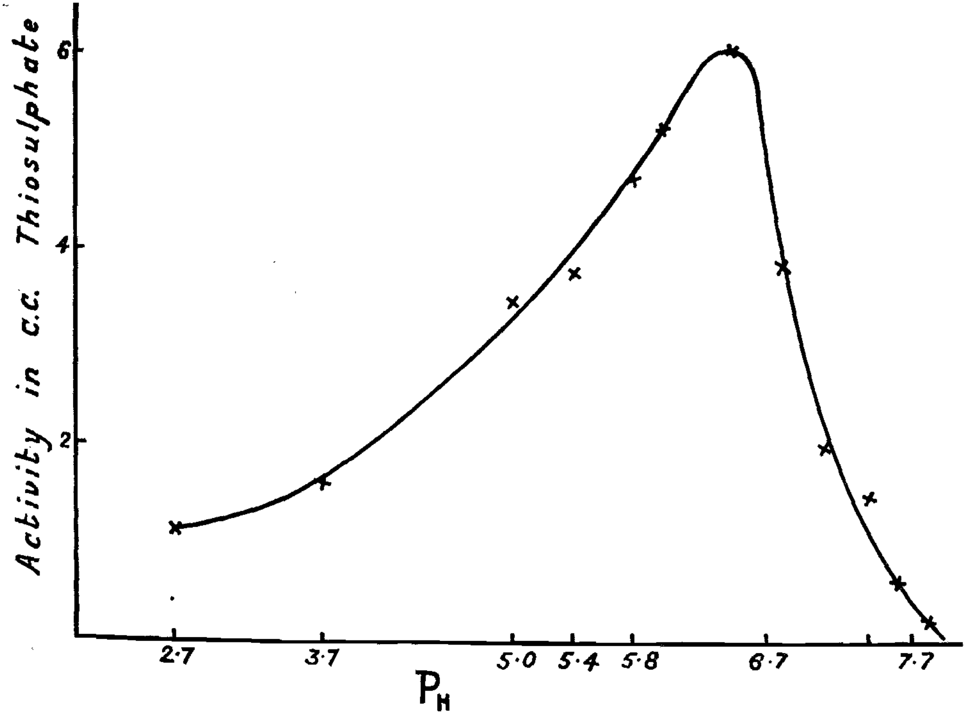


Fig. II

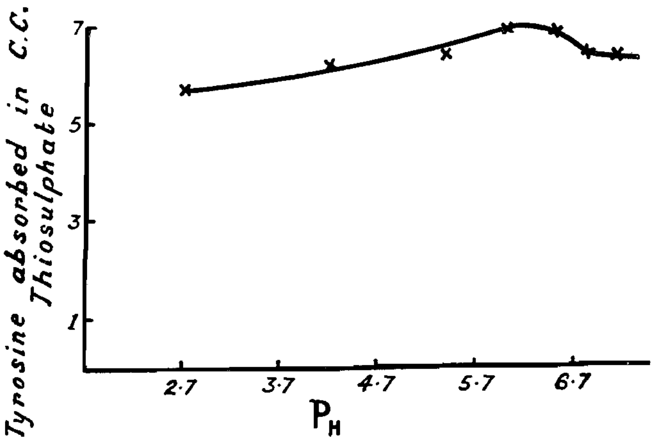


Fig. III

found by measuring the activity of the enzyme at different hydrogen-ion concentrations, from 2·7 to 6·5, in Walpole's acetate buffer and in Palitzsch's borate buffer for the range 6·7–8·0. The results are given in Table II.

TABLE II.

The reaction mixture contained 10 c.c. buffer solution, 25 c.c. tyrosine solution (0·1 per cent.), 10 c.c. distilled water, 5 c.c. dialysed enzyme extract and 5 c.c. toluene. The temperature was 30° and the duration of experiment 4 hours.

P_H	2·7	3·7	5·0	5·4	5·9	6·0	6·5	6·8
Tyrosine oxidised (c.c. thiosulphate) ...	1·1	1·6	3·5	3·8	4·8	5·3	6·1	3·9
P_H	7·1	7·4	7·6	7·8				
Tyrosine oxidised (c.c. thiosulphate) ...	2·0	1·5	0·5	0·2				

The enzyme from *Dolichos lablab* is more stable towards acidity. Raper found that the potato enzyme was inactive below P_H 4·0 and explains this on the possibility that the enzyme is bound to a protein which is precipitated at this P_H. On the other hand the enzyme occurring in *Dolichos* is active even at P_H 2·7, though the activity is considerably diminished. Another interesting fact to be noted is that we found the absorption of tyrosine by 'Norit' is also at a maximum at P_H 6·0–6·5. The results are given in Table III and Figure III.

TABLE III.

The mixture contained 20 c.c. buffer solution, 50 c.c. tyrosine solution (0·1 per cent.), 0·5 gm. 'Norit' (passed through 150 mesh sieve) and 30 c.c. distilled water. It was shaken and kept in an incubator at 32° for 24 hours to attain equilibrium, then was filtered and the tyrosine left in solution estimated in 20 c.c. samples.

P_H	2·7	4·2	5·4	6·0	6·8	7·1
Tyrosine adsorbed (c.c. thiosulphate) ...	5·7	6·2	6·4	6·9	6·4	6·4

Tyrosine is isoelectric over the range 4·5–6·5 and it is evident from our adsorption experiments and from those of Abderhalden and Fodor with several amino-acids that these are best adsorbed at their isoelectric points. The experiments indicate that oxidation is probably greater in the case of undissociated tyrosine. Experiments are also in progress to measure the adsorption of tyrosine on tyrosinase at various hydrogen-ion concentrations.

The Isoelectric Point of Tyrosinase.—This constant has not hitherto been determined and is of great theoretical importance with regard to the nature of enzyme action, whether viewed from Michaelis' electro-chemical dissociation theory or from the adsorption theory. Qualitative experiments made by us indicate that the isoelectric point of tyrosinase prepared from *Dolichos lablab* approximates to P_H 6·2. The enzyme solution (10 c.c.) was usually mixed with 5 c.c. of the buffer having the required P_H and placed in the middle chamber of a Michaelis cataphoresis apparatus of pyrex glass. The stopcocks were closed and the two side limbs filled with buffer solution (5 c.c. diluted with 10 c.c. of conductivity water). A platinum foil dipping in the buffer solution in contact with solid monosodium phosphate (Sherman, *J. Amer. Chem. Soc.*, 1924, **46**, 1711) served as anode, and copper in copper sulphate was used as cathode. The electrodes were connected to the side limbs of the U-tube by agar junctions. The current (220 volts) was switched on and the stopcocks opened; migration was allowed to proceed for 18 hours when the solution in the two limbs was tested for activity with tyrosine solution.

TABLE IV.

P_H	Activity of Solution	
	Anode	Cathode
6·5	Red in few minutes.	Very pale rose after 4 hours.
6·0	...	Reddens quickly.
5·57	...	Do.
7·36	Red in few minutes.	...

The results indicate that the isoelectric point lies between P^H 6·0–6·5. In the previous section it was shown that tyrosinase acts best at P_H 6·5 when tyrosine is least dissociated and most easily adsorbed. The experiments of Frumkin (*Nature*, 1926, **117**, 790; *Biochem. Z.*, 1927, **182**, 220) also clearly indicate that neutral molecules are most easily adsorbed near the isoelectric point of the adsorbent. Wright and Rideal (*Trans. Faraday Soc.*, 1928, **24**, 530) have recently shown that the decomposition of hydrogen peroxide at a variety of surfaces is greatest near the isoelectric points of the surfaces studied. The migration experiments on tyrosinase show that the isoelectric point is not far removed from the optimum P_H and hence tyrosinase action is a case of adsorption catalysis.

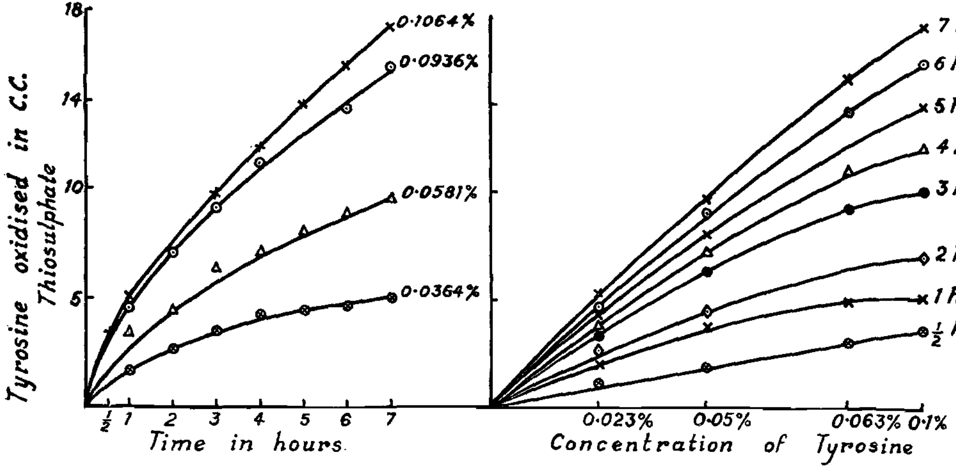

Tyrosine oxidised in C.C. Thiosulphate
18
14
10
5
½ 1 2 3 4 5 6 7
Time in hours
0·1064%
0·0936%
0·0581%
0·0364%
0·023% 0·05% 0·063% 0·1%
Concentration of Tyrosine
7 hrs
6 hrs
5 hrs
4 hrs
3 hrs
2 hrs
1 hr
½ hr

Fig. IV

The Kinetics of tyrosinase action.

The next point of interest was to study the kinetics of tyrosinase action. The effect of substrate concentration, enzyme concentration, P_H and temperature have been investigated.

The effect of substrate concentration.—The velocity of the reaction was measured for substrate concentrations varying from 0·0364 to 0·1060 per cent. of tyrosine. As tyrosine is sparingly soluble in water higher concentrations could not be tried. The results are given in Tables V and V A and Figure IV.

TABLE V.

The mixture contained 20 c.c. of buffer solution, 20 c.c. of dialysed enzyme extract and four amounts of tyrosine, (1) 0·2128 gm. (2) 0·1871 gm. (3) 0·1162 gm. and (4) 0·0728 gm., each in 160 c.c. of water, 20 c.c. of toluene being added to each portion.

Time in minutes	Tyrosine oxidised in c.c. thiosulphate			
	1	2	3	4
30	3·3	3·2	1. 8	1· 2
60	5·2	4·8	3·55	1· 8
120	7·1	...	4· 5	2· 6
180	10·0	9·1	6· 4	3· 4
240	12·0	11·1	7· 0	4·05
300	14·0	...	8· 1	4· 2
360	16·0	13·7	9· 1	4· 6
420	17·6	15·8	9· 7	5· 0
Initial thiosulphate value of the tyrosine-tyrosinase mixtures ...	23·1	20·3	12 6	7·9

TABLE V A.

Time in minutes	Velocity coefficient			
	1	2	3	4
30	0·0051	0·0057	0·0051	0·0055
60	0·0042	0·0045	0·0054	0·0043
120	0·0031	...	0·0036	0·0033
180	0·00315	0·0033	0·0039	0·0031
240	0·00305	0·0033	0·0034	0·0030
300	0·0031	...	0·0034	0·0025
360	0·0033	0·0031	0·0036	0·0024
420	0·0034	0·0036	0·0035	0·0024
Time for half change ...	3·75 hrs.	3·65 hrs.	3·6 hrs.	4 hrs.

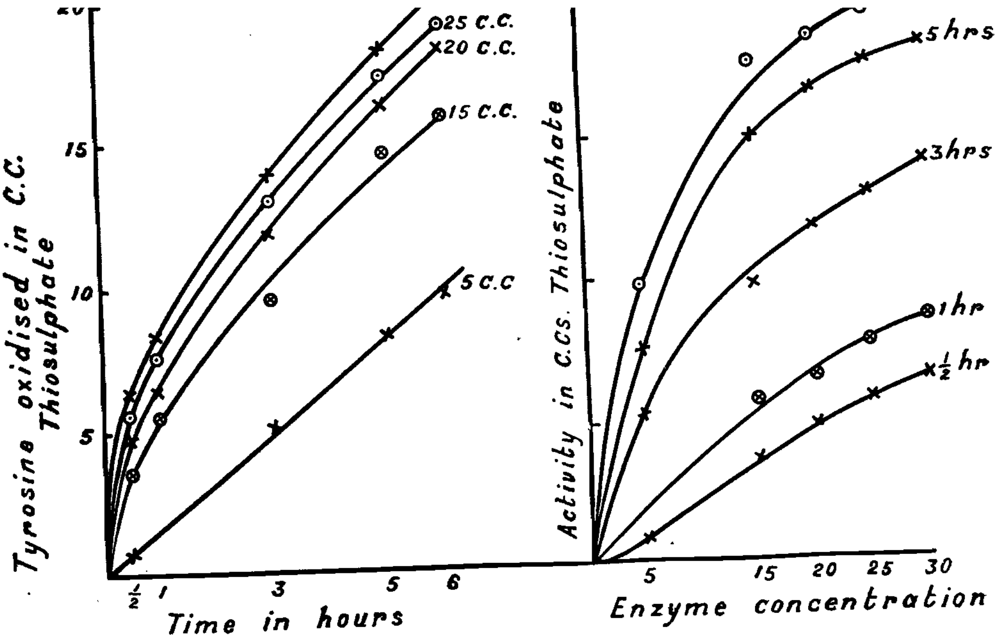
Raper (*loc. cit.*) working with potato tyrosinase found that the reaction was unimolecular, that the velocity coefficients fluctuate during the experiment showing slight decrease during the oxidation of one-half the tyrosine followed by a larger decrease of the remainder. Our results also indicate that the reaction is unimolecular, but that the decrease in the value of the coefficient is significant in the earlier stages of the reaction and later the velocity coefficient is almost constant or fluctuates about the mean value. In the case of the 0·0364 per cent. solution however the decrease is somewhat rapid at first and then diminishes slowly but continuously. The time of half change as found from the curves also suggests a unimolecular reaction. Enzyme action can be considered as a case of adsorption catalysis, and decomposition is preceded by adsorption. At higher substrate concentrations the substance decomposed at the enzyme surface is more quickly replaced than at very low concentrations and this explains the continuous fall of the coefficient at very low concentrations. If we consider the coefficients after 3 hours, those obtained at lower concentrations, i.e., 0·0936 and 0·0581 are slightly greater than at 0·1064, again suggesting an adsorption process. In view of the small amount of tyrosine present in each sample and the number of operations involved in each experiment, the divergencies are not greater than experimental errors.

Kinetics at different enzyme concentration.—The results obtained with different enzyme concentrations are given in Tables VI and VI A and Figure V.

TABLE VI.

The mixture contained 15 c. c. of buffer solution (P_H 6·5), 100 c. c. of tyrosine solution (0·15 per cent.) with enzyme solution and water in the quantities (1) 5 and 30 c. c., (2) 15 and 20 c. c., (3) 20 and 15 c. c., (4) 25 and 10 c. c., (5) 30 and 5 c. c., toluene (15 c. c.) being added to each portion : the temperature was 25°.

Time in minutes	Tyrosine oxidised in c. c. thiosulphate				
	1	2	3	4	5
30	0·8	3·7	4·7	5·7	6·5
60	...	5·6	6·4	7·6	8·2
180	5·2	9·7	11·9	13·1	14·0
300	8·2	14·9	16·3	17·5	18·1
360	9·7	15·9	18·1	18·9	20·0
Initial thiosulphate value of the tyrosine-tyrosinase mixtures	22·7	22·6	22·7	22·9	23·2



Tyrosine oxidised in C.C. Thiosulphate
5
10
15
25 C.C.
20 C.C.
15 C.C.
5 C.C
½
1
3
5
6
Time in hours
Activity in C.CS. Thiosulphate
5 hrs
3 hrs
1 hr
½ hr
5
15
20
25
30
Enzyme concentration

Fig. V

vai..
tyrosin.
tyrosinas.
mixtures

TABLE VI A.

Time in minutes	Velocity coefficients 1	2	3	4	5
30	0 00598	0·00595	0·00772	0·00953	0·011
60	...	0·00474	0·00551	0·00671	0·00726
180	0·00144	0·00311	0·00412	0·00471	0·00513
300	0·00149	0·00359	0·00382	0·00487	0·00504
360	0·00155	0·00337	0·00443	0·00484	0·00524
Average of last three	0·00149	0·00336	0·00412	0·00481	0·00514

Time in minutes ...	30	180	300	360
$k = \frac{10\,x}{t}$ ...	0·267	0·289	0·273	0·270

While in the case of varying substrate concentration the velocity constant is almost independent of the tyrosine concentration (within the limits investigated) there is a definite increase in the value of the constant with increasing enzyme concentration. This is more marked with lower concentrations, and is to be expected because at the higher concentrations more than one-half the tyrosine is oxidised within 90 minutes. Another point to be observed is that at the lowest concentration the velocity coefficients show an increase and the coefficients for a zero order reaction fit well. It is interesting in this connection to mention that Arrhenius (*Z. Anorg. Chem.*, 1923, **36**, 456) believed that all enzyme reactions are of the zero order type. At very low enzyme concentrations, the quantity of substrate being relatively large, that part decomposed at the enzyme surface is almost immediately replaced from that in solution, so that the quantity acted upon is directly proportional to the time of action. This occurrence in tyrosinase action again suggests that adsorption plays an important role in the action of this enzyme.

Kinetics at different hydrogen-ion concentrations.—Raper has worked at three different hydrogen-ion concentrations and found the largest fall in the value of the coefficient at P_H 6·0; he believes that the acid has precipitating action on the enzyme. Our experiments at three different hydrogen-ion concentrations, 4·99, 6·52 and 7·36 give a different aspect of the phenomenon. The coefficients at P_H 4·99 show a decrease after 2 hours; at P_H 7·36 the coefficients gradually increase

and the reaction assumes an autocatalytic character. This is well illustrated by Tables VII, VII A and VII B and Figure VI.

TABLE VII.

Influence of P_H *on the kinetics.*

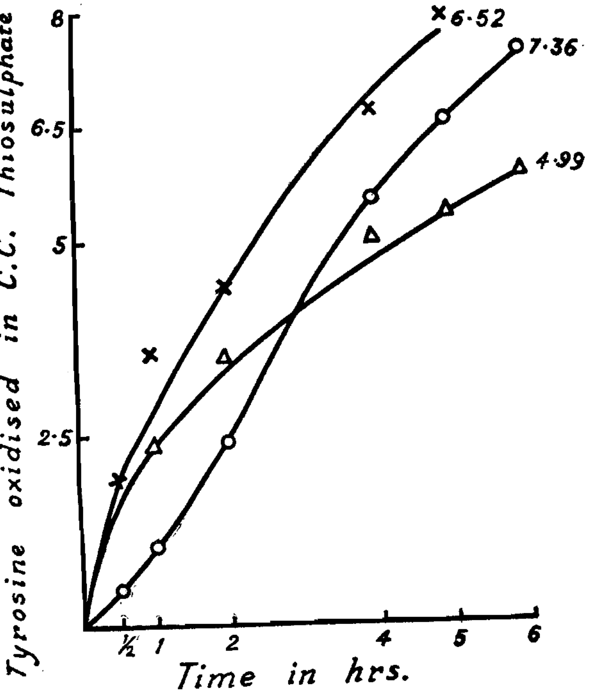
Composition of the reaction mixture : temperature, 25°.

P_H	0·0666 per cent. Tyrosine	Buffer	Water	Toluene	Enzyme solution
4·99	150 c. c.	20 c. c.	10 c. c.	20 c. c.	20 c. c.
6·52	do.	do.	do.	do.	do.
7·36	do.	do.	do.	do.	do.

Time in minutes	Tyrosine oxidised in c.c. thiosulphate		
	P_H 4·99	P_H 6·52	P_H 7·36
30	1·0	1·9	0·6
60	2·0	3·8	1·2
120	3·6	4·4	2·4
240	5·2	6·5	5·7
300	5·5	8·0	6·7
360	5·9	...	7·5

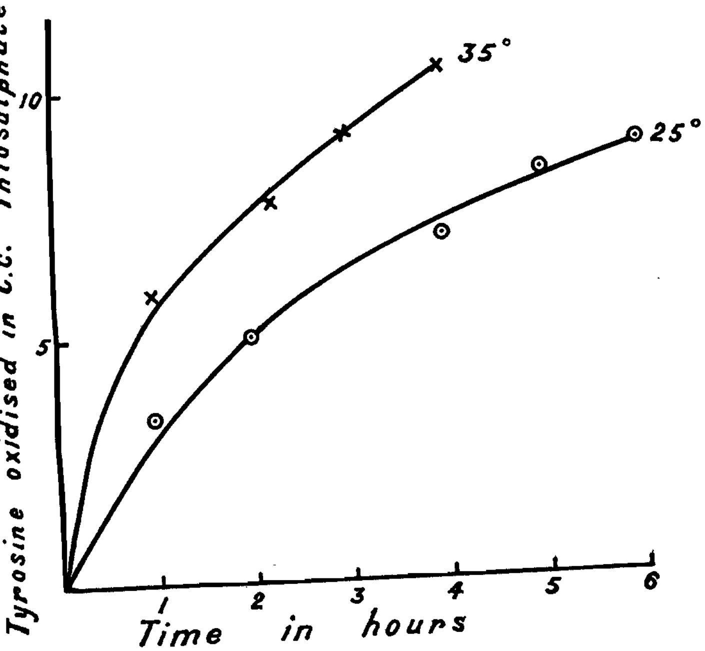
TABLE VII A.

Time in minutes	Velocity coefficients		
	P_H 4·99	P_H 6·52	P_H 7·36
30	0·00298	0·00590	0·00176
60	0·00312	0·00612	0·00180
120	0·00305	0·00393	0·00191
240	0·00235	0·00314	0·00277
300	0·00211	0·00383	0·00283
360	0·00195	...	0·00277



Tyrosine oxidised in C.C. Thiosulphate
8
6·5
5
2·5
6·52
7·36
4·99
½
1
2
4
5
6
Time in hrs.

Fig. VII



Tyrosine oxidised in C.C. Thiosulphate
10
5
35°
25°
1
2
3
4
5
6
Time in hours

Fig. VII

TABLE VII B.

Time in minutes.	30	60	120	240	300	360
$k = x/t$; P_H 7·36	0·020	0·020	0·020	0·024	0·022	0·021

The initial thiosulphate value of the reaction mixture was 11·7 c.c.

It must also be mentioned that Raper observed (with potato tyrosinase) a slight decrease at P_H 8·0 and he attributes this to the possible adsorption of the enzyme on the melanin formed.

Effect of Temperature.—As the temperature coefficient of a reaction is an important factor this was determined. The reaction was conducted at two different temperatures 25° and 35°. The results are given in Table VIII and Figure VII.

TABLE VIII.

The reaction mixture contained 100 c. c. tyrosine (0·08 per cent.), 32 c. c. buffer at P_H 6·5, 12 c. c. water, 16 c. c. enzyme solution and 16 c. c. toluene.

Time in minutes	...	60	120	135	180	240	300	360
Tyrosine oxidised	25°	3·4	5·2	...	...	6·9	8·3	8·7
(c. c. thiosulphate)	35°	5·9	...	7·3	9·0	10·2	...	...

Initial thiosulphate value of the reaction mixture was 10·2 c. c.

Time in minutes	Velocity coefficients		
	k_{25}	k_{35}	k_{35}/k_{25}
60	0·00675	0·0147	2·13
120	0·00593	...	...
135	...	0·00984	1·66
180	...	0·00851	...
240	0·00469	0·0082	1·83
300	0·00412	...	...
360	0·00423	...	...
	Average of last three 0·00435	Average of last two 0·00835	Average of last two 1·75

The coefficients at 35° are greater than those at 25° and the ratio k_{35}/k_{25} is approximately 2. The heat of activation calculated according to the Arrhenius equation :—

$$A = \frac{\log k_2 - \log k_1}{0 \cdot 4343} \times R \frac{T_2 T_1}{T_2 - T_1}$$

is 12,250 calories.

It is also interesting to note (from the results for 35°) that the reaction can proceed to completion for the tyrosine oxidised in 4 hours is 100 per cent.

Influence of External Factors.

Temperature.—The thermostability of the enzyme in aqueous solution was next investigated. That plant tyrosinase preparations are moderately stable to heat has been noticed by Bertrand as well as by Haehn. Equal quantities of the enzyme solution were exposed to temperatures of 65–70°, 80° and 95°, for half an hour. At the end of this period the solutions were centrifuged to remove suspended and heat-coagulated matter and their activities compared with that of an untreated solution. The results are given in Tables IX and IX A.

TABLE IX.

The reaction mixture contained 25 c. c. tyrosine (0·1 per cent.), 10 c. c. buffer at P_H 6·5, 10 c. c. water, 5 c. c. enzyme solution and 5 c. c. toluene.

No.	Exposed to	Observation
1	(Untreated)	Clear solution.
2	65–70°	Slightly opalescent.
3	80°	Coagulates ; white precipitate.
4	95°	Precipitate larger than in 3.

TABLE IX A.

Exposed to	Activity in c. c. thiosulphate
(Untreated)	5·7
65–70°	5·0
80°	Light rose coloration after 4 hours
95°	No action

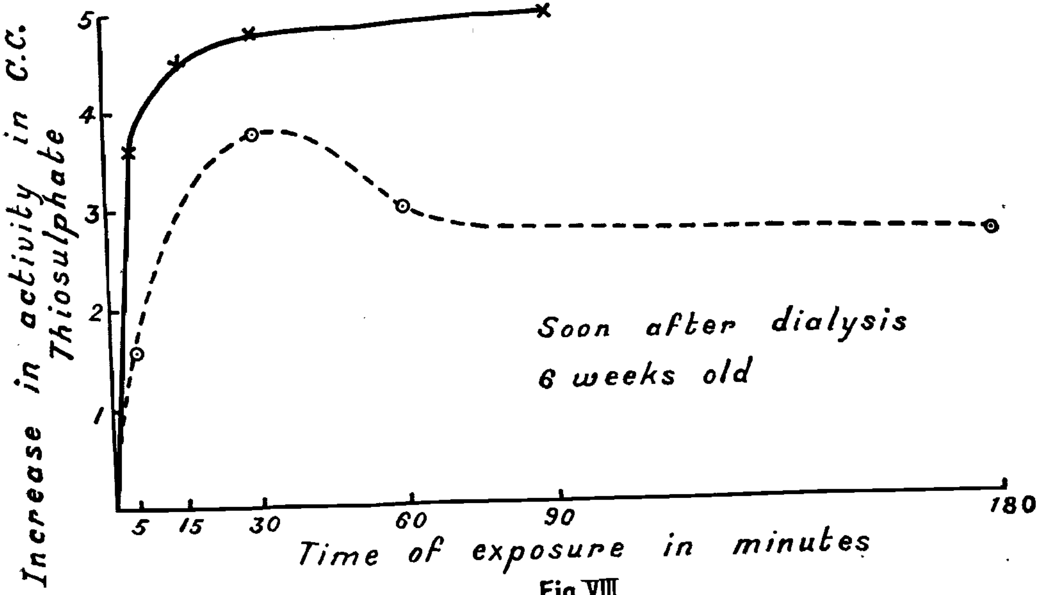

Increase in activity in C.C. Thiosulphate
5
4
3
2
1
5
15
30
60
90
180
Time of exposure in minutes
Soon after dialysis
6 weeks old

Fig. VIII

The duration of the reaction was two hours and the temperature 25°.

The results show that the enzyme is fairly stable to 70°, although its activity is slightly diminished; it is almost destroyed in 30 minutes at 80° and completely at 95°.

Action of ultra-violet light.—According to Green, Schmidt-Nielsen and Jodlbauer ultra-violet rays have a destructive action on all enzymes. Agulhon (*Compt. rend.*, 1911, **153**, 979) distinguishes the effect of the rays in presence and absence of oxygen. The effect of exposure to the rays from a quartz mercury lamp (110 volts) was tried on the *Dolichos* tyrosinase in aqueous solution, and the activity found to be increased. The results are given in Table X and Figure VIII.

TABLE X.

Time in minutes	Activity in c. c. thiosulphate	Increase in activity
0	4·4	...
5	8·0	3·6
15	8·9	4·5
30	9·2	4·8
90	9·4	5·0

TABLE X A.

Time in minutes	Activity in c. c. thiosulphate	Increase in activity
0	7·1	.
5	8·7	1·6
30	10 9	3 8
60	10·1	3 0
180	9·8	2·7

The enzyme solution was exposed to the action of light from the quartz mercury lamp in silica flasks. Table X shows results obtained with an enzyme solution exposed to the action of the rays soon after extraction and dialysis; XA the results from a solution 6 weeks old. The duration of the experiment in the first case was 3 hours and in the second case 3 hours 20 minutes. The results

clearly show an increase in activity even in the case of the aged solution, and that after reaching a maximum it slightly diminishes.

It is known that ultra-violet rays have two different biological effects, a stimulative effect and a lethal one. Stimulation is caused by rays from the limit of the visible spectrum to about 290 $\mu\mu$ (2900 A°). This corresponds to the ultra-violet component of the solar spectrum. Waves shorter than 290 $\mu\mu$ (2900 A°) exert a lethal effect.

It was observed by Lindner (*Woch. Brau.*, 1922, **39**, 166) that exposure to ultra-violet rays considerably increased the fermentation of dextrose by yeast, and that exposure of the cells in a shallower layer produced the opposite effect. Clark (*Amer. J. Physiol.*, 1922, **61**, 72; *Amer. J. Hyg.*, 1922, **2**, 322) supports the theory that action of light on organic compounds is an emission of electrons from the substance affected. Howell found that precipitation of fibrinogen by ultra-violet rays is inhibited in the presence of hematoporphyrin.

These observations suggest a possible mechanism of the action of ultra-violet rays on tyrosinase, namely, that by emission of electrons the negative charge on the enzyme particle is considerably lowered and brought to a state when it has a very small velocity of migration in an electric field. We have indicated in previous sections of this paper that tyrosinase action is to be considered as an instance of adsorption catalysis and that its activity is greatest when the enzyme has a small negative charge. The fall in the increase in activity with longer exposures is also easily explained on the basis of this theory. Cataphoretic studies are in progress to test this. Another possible explanation is the probable higher dispersion effected by the ultra-violet due to the action of some compound present as in Howell's experiment (*Archiv. inter. physiol.*, 1921, **18**, 269). A third possibility is the formation of an activator. Another point to be considered in this connection is the probable absorption of the lethal rays before they reach the enzyme. Experiments are in progress to study the effect under standardised conditions, using pure enzyme preparations. The effect of different parts of the mercury arc spectrum, added impurities, salts, P_H, etc. are being studied.

Action of Hydrogen Peroxide.—Gessard found that hydrogen peroxide activates tyrosinase and Bach made a similar observation with potato tyrosinase; both Staub and Abderhalden found inactivation. This is of theoretical importance, for according to Oppenheimer, if Gessard's finding is confirmed, then the probability that tyrosinase is a dehydrase becomes greater. Our results are given in Table XI.

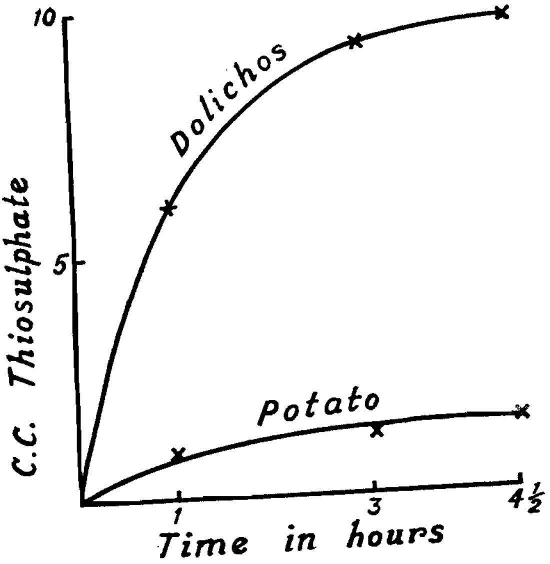


Fig. IX

TABLE XI.

Peroxide per cent.	Colour.	Activity in c.c. thiosulphate			
		Unaerated		Aerated	
		Immediate	3 hours	Immediate	3 hours
0	Red after a few minutes...	0	4·6	0	8·0
0·2	Deep red immediately ...	2·5	2·5	2·5	2·5
0·02	Deep red in a few seconds.	1·5	1·5	1·4	4·2
0·002	Red in a few seconds ...	1·5	1·5	1·5	6·3
0·0002	Red in a few minutes ...	1·0	2·7	1·0	8·9

Two series were run, one aerated and the other unaerated. No activation was observed in the unaerated samples. If readings were taken immediately after adding the hydrogen peroxide and enzyme, activation was observed, but after three hours there was practically no increase in the amount of tyrosine oxidised. In the case of aerated samples slight activation was observed at the lowest concentration. Perhaps two effects have to be taken into account, activation of some part of the reaction and destruction of the enzyme by the hydrogen peroxide.

Comparison with the enzyme prepared from Potato.—Since much of the previous work involved tyrosinase prepared from the potato, the activity of this was compared with that of the enzyme prepared from *Dolichos*. The best potatoes available in the market were minced and the juice obtained with a Buchner press at 1·5 tons, centrifuged to remove starch and dialysed in collodion bags under slight pressure against flowing distilled water. The activity of the dialysed solution was then compared with that of an enzyme preparation obtained from *Dolichos lablab* under identical conditions, the solid content of the two solutions being the same. The results are given in Table XII and Figure IX.

TABLE XII.

Time in minutes	Tyrosine oxidised in c.c. thiosulphate	
	Dolichos	Potato
60	6·0	1·0
180	9·2	1·2
270	9·5	1·4

The activity of the enzyme prepared from the potato is very much lower than that obtained from *Dolichos* under the same conditions. Haehn (*Biochem. Z.*, 1920, **105**, 169) has stated that potato tyrosinase loses its activity on dialysis and that addition of boiled potato-juice restores the activity. We did not notice this and the undialysed juice was not more active than the dialysed one. Raper (*Biochem. J.*, 1923, **17**, 454) has drawn attention to the fact that the activator is present only in the juice of fresh potatoes, and the potatoes we used were not fresh. According to Abderhalden (*Fermentforschung*, 1926, **8**, 479) the enzyme prepared from the potato is of poor activity, is stable only for a short period and is rich in ballast material and impurities. On the other hand our preparations from *Dolichos* are very active, and preserve their activity in aqueous solution over a considerable length of time.

Purification of the enzyme.—Apart from ordinary dialysis and precipitation with alcohol no purification study has been attempted by earlier workers. The increase in activity obtained with diastase (Narayanamurti and Norris, *J. Ind. Inst. Sc.*, 1928, **11A**, 134) by electrodialysis led us to try this method, which ought to be valuable in the study of tyrosinase as Haehn has claimed (*Biochem. Z.*, 1920, **105**, 169) that tyrosinase could be separated into two components by dialysis as well as by ultra-filtration; also that the component which diffuses in dialysis and forms the filtrate in ultra-filtration is a thermostable, inorganic activator. Raper has partially confirmed Haehn's finding, but only in the juice of fresh potatoes; he also believes that this activator is organic.

It has been frequently suggested that traces of iron or manganese are essential for the activity of oxidases. This is suggested by Fenton's reaction. As remarked by Bayliss, the investigations of Willstätter and Pollinger however suggest caution, for they found that the activity of peroxidase preparations was not in proportion to the iron-content. Since electrodialysis will remove all inorganic impurities, the role of manganese and iron in the inorganic form can be easily decided.

The crude meal-extract after filtration was dialysed for three days against running distilled water to remove most of the protein and then one portion was put in the electro-dialyser and another submitted to ordinary dialysis. The electro-dialysis was at 30 volts. By having the anode space thrice as large as the cathode space and allowing a very low current to pass, acidification was avoided. Soon after the electro-dialysis was finished, the solutions were compared for their activities on tyrosine. Two sets of experiments have been conducted, one with parchment and another with collodion membranes.

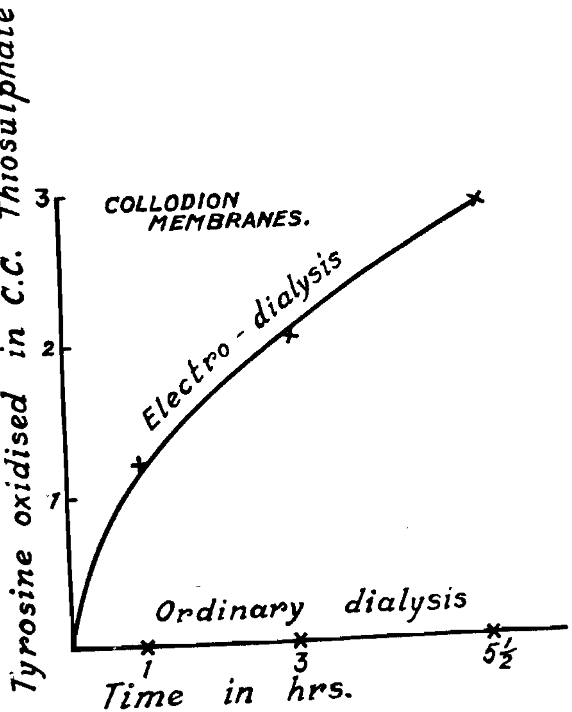

COLLODION MEMBRANES.
Electro-dialysis
Ordinary dialysis
Tyrosine oxidised in C.C. Thiosulphate
Time in hrs.
1
2
3
1
3
5½

Fig. X

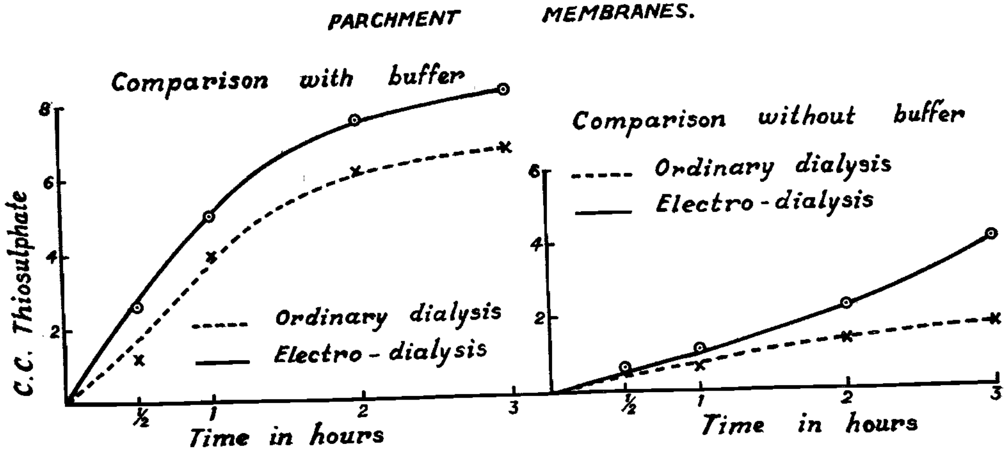

PARCHMENT MEMBRANES.
Comparison with buffer
Comparison without buffer
Ordinary dialysis
Electro-dialysis
Ordinary dialysis
Electro-dialysis
C.C. Thiosulphate
Time in hours
Time in hours

Fig. XI

In both these cases the electrodialysed enzyme was more active, with buffer or without buffer. These experiments indicate that inorganic salts do not play any great part in the action of this enzyme and that if an activator is present it must be in the colloidal condition, or at least bound to a colloidal surface (c. f. Onslow, *Biochem. J.*, 1928, **22**, 1327). The results are given in Tables XIII and XIII A and Figures X and XI.

TABLE XIII (Collodion membrane).

The reaction mixture contained 50 c. c. tyrosine (0·05 per cent.). 50 c. c. enzyme solution and 10 c. c. toluene. The temperature was 34·6°.

Time in hours	Tyrosine oxidised in c. c. thiosulphate	
	Electro-dialysis	Ordinary dialysis
1	1·2	...
3	2·0	...
5·5	2·8	Light rose coloration

TABLE XIII A.

(Parchment membranes).

Composition of the reaction mixture.

No.	Method of preparation	Tyrosine 0·1 per cent.	Buffer P_H 6·52	Water	Toluene	Enzyme solution
1	Electro-dialysis	50 c. c.	10 c. c.	20 c. c.	10 c. c.	20 c. c.
2	Ordinary dialysis	50 c. c.	10 c. c.	20 c. c.	10 c. c.	20 c. c.
3	Electro-dialysis	50 c. c.	...	30 c. c.	10 c. c.	20 c. c
4	Ordinary dialysis	50 c. c.	...	30 c. c.	10 c. c,	20 c. c

Temperature of the reaction 25°.

Time in minutes	Tyrosine oxidised in c. c. thiosulphate			
	With buffer		Without buffer	
	Electro-dialysis	Ordinary dialysis	Electro-dialysis	Ordinary dialysis
30	2·6	1·2	0·6	0·3
60	5·0	3·9	1·1	0·6
120	7·5	6·1	2·2	1·3
180	8·2	6·6	4·0	1·6

Ultra-filtration.—These experiments were conducted to seek ɜvidence for the assumption that tyrosinase is a mixture of components ɪnd also to test Haehn's statements (*Biochem. Z.*, 1920, **105**, 159) that .he ultra-filtrate is necessary for the action of the enzyme. Experi- nents were made with an improved form of apparatus described by 3rukner (*Z. Ver. Deut. Zucker Ind.*, Band **76**, *Technischer Teil*, /ol. 837, June 1926; Dissertation, University of Gottingen) using Zsigmondy's ultra-fine filters as well as parchment. Compressed air vas used for pressure and the experiments were made without stirring. Filtrates collected up to a pressure of 50 atmospheres were ound to have no activity; on the filter there was much precipitate vhich on dispersion in water was found to be active. The residual iquid on the filter was slightly more active than the untreated solution; ddition of ultra-filtrate did not cause any increase in activity. These xperiments clearly show that the tyrosinase from *Dolichos lablab* does ıot lose activity on ultra-filtration: they also indicate the probable bsence of any activator and that if present it must be colloidal or ·ound to a colloidal carrier; further that the enzyme is of a coarse ispersity. The possibility of obtaining the solid enzyme free from all rystalloidal impurities is also clearly indicated. About 150 c.c. of the ialysed enzyme solution were put in the ultra-filtration apparatus and he filtrates collected at pressures of 25 and 50 atmospheres. The esults are given in Table XIV.

TABLE XIV.

No. of filtrate	Pressure at which collected	Volume in c.c.
1	25 atm.	28
2	50 atm.	28
3	50 atm.	52
esidual liquid on the filter ...	... ...	38
Total ...	... ...	146

The sticky black precipitate on the filter was dispersed in 20 c.c. of distilled water and the activities of the several fractions and the dispersed solid compared with that of the untreated solution. The next day the residual liquid (preserved in the ice-box) was found to have deposited a large amount of solid; the clear upper layer of liquid was however still very active.

TABLE XIV A.

Fraction collected at	Activity in c. c. thiosulphate
(Untreated)	7·5
25 atm.	0
50 atm.	0
50 atm. (2nd fraction)	0
Residual liquid	7·6
The dispersed precipitate	7·7

The concentration of tyrosine was 0·05 per cent. and the reaction was carried out at P_H 6.52 and temperature 30°.

Ultra-filtration with parchment membranes.—The experimental details were the same as with Zsigmondy's ultra-fine filter. About 135 c.c. of the enzyme solution were used. The results are given in Tables XV and XV A.

TABLE XV.

No. of filtrate	Pressure at which collected	Volume in c. c.
1	25 atm.	21
2	50 atm.	25
3	50 atm.	76
4 Residual liquid on the filter ...	... ...	7
Total ...	... ...	129

The precipitate on the filter was dispersed in 25 c.c. of water and the activities of the various fractions compared with a sample of the untreated solution.

TABLE XV A.

Fraction collected at	Activity in c. c. thiosulphate
(Untreated)	7·6
25 atm.	0
50 atm.	0
50 atm.	0
Residual liquid	5·3
The dispersed precipitate	10·0

Effect of adding the Ultra-filtrate to the residual liquid.

TABLE XVI.

—	Residual liquid from Zsigmondy filter (5 c.c.) + 5 c.c. water.	Residual liquid from Zsigmondy filter (5 c.c.) + 5 c.c. ultra-filtrate.
Activity in c.c. thiosulphate ...	9·3	9·2

SUMMARY.

Active preparations of tyrosinase stable in aqueous solution for a considerable length of time have been obtained from the ungerminated seed of *Dolichos lablab*.

The solubility relations of the enzyme indicate its colloidal nature.

The optimum P_H and the isoelectric point of the enzyme are in the neighbourhood of 6·5.

The kinetics of tyrosinase action under varying conditions of substrate concentration, enzyme concentration, hydrogen-ion concentration and temperature have been investigated. That the reaction can proceed to completion is also indicated.

The enzyme in aqueous solution is stable up to 70°.

Tyrosinase is activated by exposure to the rays from a quartz mercury arc.

The influence of hydrogen peroxide in the action of the enzyme has been studied.

Preparations practically free from electrolytes have been obtained for the first time by subjecting tyrosinase to electro-dialysis, by which tyrosinase is considerably increased in activity.

Evidence based on electro-dialysis and ultra-filtration experiments shows that contrary to Haehn's view inorganic salts are not necessary for the action of this enzyme, and that, if any co-enzyme is present, it must be colloidal, or associated with a colloidal carrier.

We desire to express our indebtedness to Prof. R. V. Norris for the keen interest he has taken in this work.

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THE ACTIVATED SLUDGE PROCESS OF SEWAGE TREATMENT.

Report on the working of the Plant at the Indian Institute of Science, Bangalore.

By N. Swaminathan.

In July, 1922, a new plant for the disposal of sewage by the activated sludge process was installed at the Institute, and has now been treating domestic sewage proceeding from a section of the inhabitants during a period of more than five years. The process is a complete contrast to the older anaerobic methods and is a development of the biological sewage-treatment in bacteria-beds, percolating filters and the like, being aerobic treatment in open tanks. The method depends on the sewage flowing through the tanks being vigorously agitated by finely divided compressed air. The oxygen supplied, in conjunction with the ubiquitous oxidising organisms in the sewage, effects a distinct clarification of the offensive liquid admitted and precipitates all the collodial matter, yielding a valuable fertiliser. This intensive bacterial oxidation is the most up-to-date form of sewage disposal, and has been adopted at the Institute both for sanitary reasons and for large-scale biochemical investigations.

Description of Works.

The installation works on a continuous flow basis. It occupies an area of roughly 1,500 s. ft. and was constructed by a local European contractor to designs prepared by Messrs. Activated Sludge, Ltd., London. The plant was designed for 200 persons at 30 gallons per head per day, or 6,000 gallons per 24 hours. It has, however, been used for nearly 400 persons, the rate per head being about 18 gallons per day. The plant commands 14 acres of ground on which the effluent can be easily absorbed, and was brought into commission on July 10, 1922.

The works comprise tanks for aeration, settlement and re-aeration, built of masonry work and below ground level. The aeration tanks are three rectangular channels $27' \times 2' \times 3'$ to water level, subdivided into compartments by vertical baffle walls; the cubic contents are 510 c. ft., equivalent on the designed basis to 2·55 c. ft. per head,

Under present conditions it now comes to 1·36 c. ft. per head, taking the number of persons connected to the sewers at 375. The period of sewage-detention was fixed at 4 hours, but subsequently, owing to the varying rates of flow, the actual period has ranged between 3·2 and 5·5 hours. The sewage traverses the whole length of the channels to secure complete aeration and has little chance of short-circuiting; the floors are flat, and aeration is effected by rows of diffusers fitted loosely in grooves at the bottom on one side of the walls.

The settlement tank is 5′ × 5′ × 5′ and ends in a hopper bottom, the sides having a slope of 60° to the horizontal. The depth from the apex of the hopper bottom to the surface is 9′. The mixed liquid proceeds from the aeration-chamber to the centre of the settlement tank through a Clifford inlet inside a guard chamber, and is gently received by a wrought-iron eddy-bucket suspended from two rails fixed to the walls of the tank. The sludge and liquid are here separated, and the purified effluent passes over two weirs on either side of the tank. The Clifford inlet is fitted with a disc-valve normally closed, and opened only when the aeration tanks are drained into the settlement tanks.

The deposited sludge is carried by hydrostatic head from the apex of the hopper bottom through a pipe to the sludge re-aeration channel, similar to the aeration channel but narrower, and also divided into three compartments by baffle walls. At the entrance of the sludge return-pipe there is another disc-valve normally open, and closed only when the aeration tanks have to be drained into the settlement tank and no return sludge is needed in this channel. The sludge from here is raised by an air-lift into the mixing chamber to inoculate incoming sewage with the necessary bacteria. A third disc-valve at the outlet of the air-lift pipe is normally kept open, and closed when surplus sludge has to be pumped out. Another air-lift pumps the surplus sludge into a sump whence it passes through an earthenware pipe into a compost pit.

Air equipment.—The air for agitating the sewage in the tanks is derived from two compressors working alternately. A Reavell compressor working with a three phase induction motor of 3 H.P. delivers a constant supply of 15 c. ft. of free air per minute at 690 r.p.m. This motor works for 15 hours a day, i.e., from 5 p.m. to 8 a.m. A similar motor of 5 H.P. operates a belt-driven Scott compressor and delivers 40 c. ft. of free air per minute at 750 r.p.m. working for 9 hours a day, i.e., from 8 a.m. to 5 p.m. The air delivered can be measured by a curved tube manometer. From July 1922 to October 1923 the Reavell compressor was the only one in operation. After October 1923 the Scott compressor was working and consequently the air supply was increased.

Distribution of air.—The air is distributed by two cast-iron mains fixed to the tank-walls, a slide valve on each main regulating the air supply as desired in any two pairs of tanks. To the mains are attached a number of down pipes, each fixed to a diffuser in a groove at the bottom of the tank. The air-supply to any particular diffuser can be regulated also by a throttle valve. All the air-lifts are worked by compressed air, and the diffusers are of porous material cemented to shallow cast-iron plates.

Operation of the plant.—The tanks were filled and air blown in without admitting more sewage until a sample of the liquid showed good nitrification, the time being a fortnight; a small quantity of sludge was also formed during this period. The entering sewage was purely domestic, and prior to treatment was passed through a system of screens and catch-pits to remove floating solids, heavy detritus and unemulsified material generally. At the mouth of the pipe admitting sewage into the sludge tanks, another wire gauze screen prevented such material as had passed the catch-pits from entering the tanks and choking the pipes. A valve at the inlet pipe regulated the flow and diverted it either completely or partially as required: there was no recorder to gauge the quantity of sewage entering or leaving the tanks.

After complete nitrification of the first filling more sewage was admitted, oxidised and replenished, the process being repeated until there accumulated in the tanks an amount of sludge capable of purifying the sewage in a few hours. In about a month sufficient sludge had been built up, and nitrification was rapid; the Reavell compressor was working from 10 a. m. to 5 p. m. during this period. Sewage was henceforth admitted from 10 a. m. to 1 p. m. and subsequently until 5 p. m. The sludge was chocolate-brown, settled fairly rapidly, and had an earthy smell; the effluent was quite clear and non-putrefactive. Initially the amount of sludge maintained in the aeration tanks was 25 per cent., but early in September, 1922, the larvae of an insect subsequently found to be *Chironomus* rapidly developed and collected all the sludge in small clumps, some of which floated while others became attached to the walls. The floating masses proceeded to the settlement tank and thence passed with the effluent over the weirs. Thus within three days a large amount of sludge was lost. Mechanical disturbance was without effect, and the only way of removing the larvae was to add kerosine, which killed most of them at once while dissipating the red colour. After brushing the particles from the walls and adding a little more kerosine, aeration was renewed, purification of new sewage being effected in less than 24 hours in spite of the large amount of kerosine added. Thereafter the plant worked satisfactorily.

From the beginning of December, 1922, the Reavell compressor was working continuously and early in February, 1923, the plant was

treating the full flow of sewage. During this month flow-measurements were made to ascertain the total quantity entering the tanks, successive four-hour periods being kept under observation; 15-minute samples of sewage were taken and flow-measurements made by noting with a stop-watch the time taken to fill a two-gallon measure held under the sewage inlet pipe. Hourly average samples were obtained by mixing portions of the quarter-hour samples in proportion to the flow at the time. Samples of effluent were also collected every hour and an average of the hourly samples also mixed in proportion to the flow. All these samples were analysed for chlorine and for oxygen absorbed from acid potassium permanganate solution in 3 minutes. A complete analysis of the average representative sample was also made. The results are given in Chart I and Table I.

The total flow over a 24-hour period was calculated to be 6,575 gallons. From this table it appears that practically one-third of this flow reached the tanks between 7 and 11 a.m., the maximum rate of flow being 18·6 gallons per minute at 8 a. m. The flow from 11-30 p. m. to 6·30 a. m., did not exceed 0·66 gallon a minute. According to the above calculation, the volume of sewage treated in the tanks from July to December, 1922, amounted to about 436,000 gallons. The ratio of air-supply to sewage during this period was therefore approximately 2·51 c. ft. per gallon. It was however found advisable to adjust the air supply in accordance with the strength flow factor of the sewage (see Table I below) by pumping in more air during the day than at night. The designers of the plant had recommended 50 c. ft. of air per minute for this plant, but actually the sewage was receiving less than one-third of this quantity. The air-supply was purposely not increased in order to study the biological phenomena occurring in the sludge with the available supply. Though stable effluents were obtained with this deficient air-supply the sludge was found to go out of condition within a few months of treating the full flow of sewage. The amount of sludge in the tanks had increased enormously in bulk, was slow in settling and had changed from brown to grey, the whole material being sticky and gelatinous. The volume of sludge in the tanks, usually kept at 25 per cent., increased rapidly to 40 and subsequently to 60, continuing at this value for a period of several days. The reason for this was found to be deficient air supply. The volume of sewage treated before this disturbance, described as 'bulking', was noticed reached 493,000 gallons, and the volume of air per gallon of sewage was 3·3 c. ft. At the end of August in the same year the larvae previously noticed reappeared in such large numbers that the former procedure had to be repeated. The above facts being regarded as due to deficient aeration, a new Scott compressor capable of delivering 40 c. ft. of free air per minute was installed and brought into operation on October 4, 1923. It was also noticed that some sludge was never

TABLE I.

Analyses of average samples of sewage and effluent.

Date	Time	Oxygen absorbed				Chlorine		Amm. nitrogen		Alb. nitrogen		Nitrite nitrogen		Nitrate nitrogen	
		in 3 mins.		in 4 hrs.											
		S	E	S	E	S	E	S	E	S	E	S	E	S	E
14 2 23	6–30 to 11–30 a.m. ...	1·45	0·3	1·84	0·73	4·0	4·0	0·216	0·054	0·08	0·01	nil	0·003	nil	0·5
19 2 23	10–30 a.m. to 2–30 p.m.	1·33	0·26	2·59	0·55	4·31	3·73	0·18	0·033	...	0·023	nil	0·002	nil	1·0
22 2 23	2–30 to 6–30 p.m ...	...	...	5·1	1·22	4·86	4·46	0·24	0·09	0·15	0·03	nil	0·025	nil	0·35
27 2 23	6–30 p.m. to 6–30 a.m.	187	0·35	4·27	1·06	4·93	4·41	0·20	0·15	0·08	0·08	nil	trace	nil	nil
Average	......	1·55	0·303	3·45	0·89	4·52	4·15	0·209	0 082	0·103	0·035	nil	0·008	nil	0·62

S = Sewage. E = Effluent

under proper circulation by the Reavell compressor, deposits of ill-aerated sludge being observed in several chambers and producing denitrifying changes. It was anticipated that this difficulty would be removed by the new compressor.

Careful investigation into the varied aspects of the process were made and except that occasional deposits of silt choked the Clifford inlet-pipe or the return-sludge pipe or the air-lifts, interrupting work for a few hours, no trouble has arisen.

It has been stated that that there is an optimum air supply for activating the sludge in the tanks, higher or lower quantities being disadvantageous (*J. Inst. Eng., India*, 1925, **5**, 75). Experience with this plant has shown that defective air-supply tends to produce a 'sick' or 'ropy' sludge, while excessive aeration, even to the extent of 'burning', has never produced a 'bulked' sludge.

The flow of sewage was again measured in August, 1923, and in February and December of 1924. Except in the last case, where exactly the same procedure as that already described was followed, only the total flow per 24 hours was recorded. The detailed experimental results determined in December, 1924, are given in Chart I and Table II.

TABLE II.

Analyses of average sewage and effluent samples: parts per 100,000.

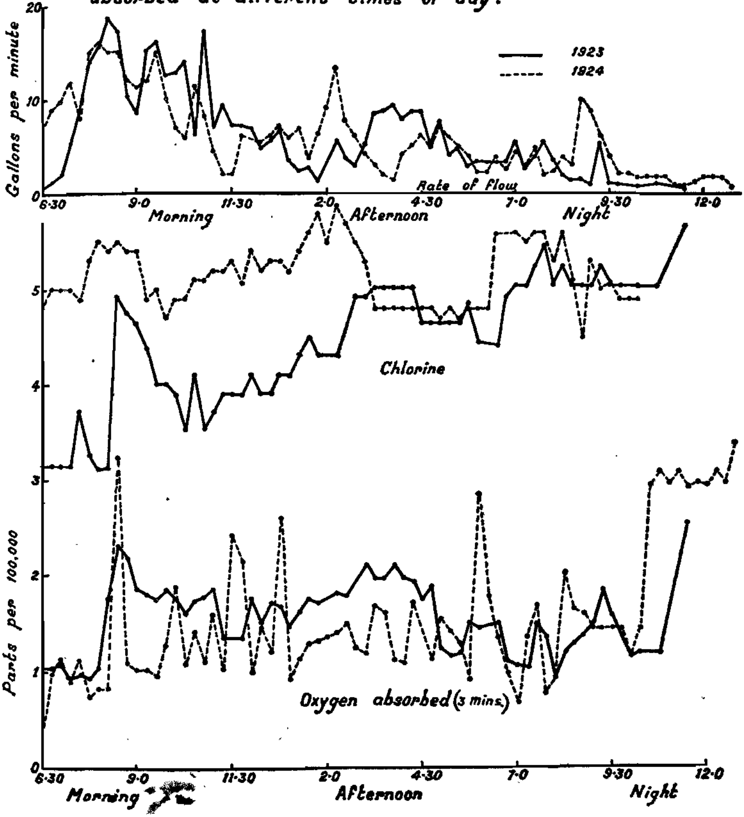
Analysis	Sewage	Effluent	Percentage purification
Oxygen absorbed from permanganate in 4 hrs.	4·23	0·48	88·6
Oxygen absorbed from permanganate in 3 mins.	1·31	0·22	...
Amm. Nitrogen	2·2	0·55	...
Alb. ,, ...	1·87	0·11	94·1
Nitrite ,, ...	nil	0·03	...
Nitrate ,, ...	nil	0·07	...
Chlorine	4·8	4·4	...

Flow Measurements.

Month	*Total flow for 24 hours*
August, 1923	.... 7,250 gallons
February, 1924	.... 4,405 ,,
December, 1924	.... 6,470 ,,

Chart I.

Rate of flow, chlorine content, and oxygen absorbed at different times of day.



The fluctuations in flow were due to (1) variation in the number of persons connected to the sewers and (2) occasional restriction of the water-supply. As before, the greatest flow was in the morning between 7 and 11, and the lowest after midnight when practically no flow could be collected. A glance at Chart I shows the characteristics of the sewage at various periods of the day in the two years. The volume of sewage treated during the several years and the ratio of air-supply to sewage were approximately as follows :—

Year	Total volume in gallons	Air supply in c.ft. per gallon
1922	436,200	2·51
1923	2,102,400	4·39
1924	1,939,800	5·22
1925	2,354,500	5·43

It has been found that, although the ratio of air supply per gallon of sewage treated seems to be very high in the last three years, the circulation of sludge in the tanks was thoroughly effected only when this volume of air was maintained. Occasionally the Scott compressor had to be stopped for short periods owing to the heat generated, when the small compressor was allowed to work; on such occasions we noticed the remarkable difference in the circulation in the sludge. With deficient air-supply the sludge was not maintained in proper contact with the sewage, and purification was incomplete. Various forms of organic life also developed and consequently the sludge lost condition.

Initially 25 per cent. of sludge was kept in the aeration tanks, but subsequent experiments showed that 10–15 per cent. gave results quite as good. Occasionally, with as low a quantity as 8 per cent., satisfactory purification was attained.

The plant was stopped and re-started on two occasions, once in July, 1924 and again in February, 1926. Each time nitrification began more rapidly than previously, being induced in a week the first time and in the second case within 48 hours. Compressor-trouble delayed nitrification in July 1924, but in February, 1926, there was no such difficulty and nitrification began more rapidly. The sludge was also built up in a few days. In both cases the full flow could be successfully treated within a fortnight, and thereafter the plant has worked continuously with only one or two short stops due to failure of current or other fortuitous circumstances.

Chemical control of the process.—Speaking generally, the chemical changes of the sewage consist in degradation of complex protein matter into simple nitrogenous substances. A chemical analysis of the sewage and effluent reveals the character of the sewage treated and the degree of purity attained. The following table gives the figures obtained for the raw sewage and of the effluent from the plant. The results in both cases are averaged from a large number of analyses.

TABLE III.

Analysis of sewage and effluent: parts per 100,000.

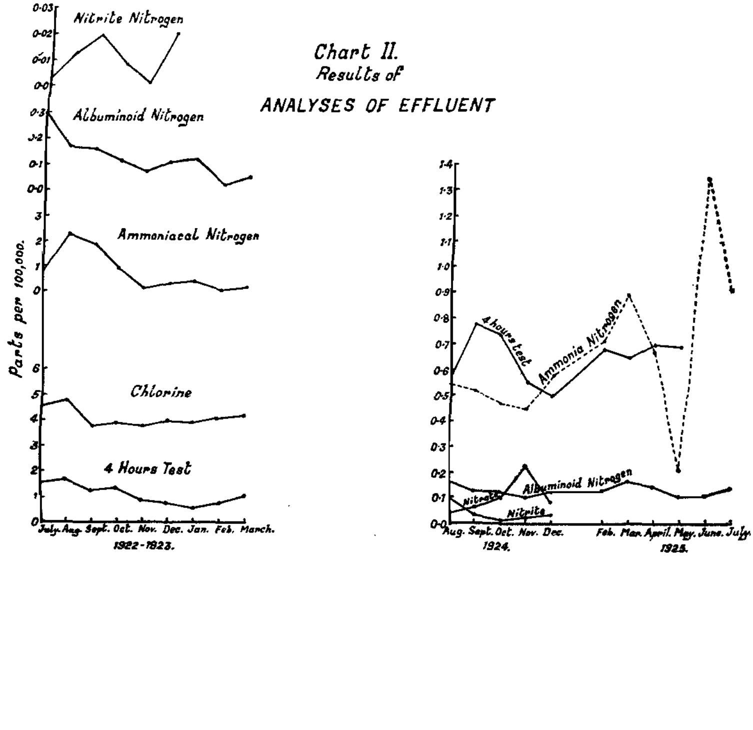
Head of analysis	*Crude sewage* 1	2	3	*Effluent* 1	2	3
Ammoniacal nitrogen ...	1·68	2·44	3·25	0·76	0·51	0·75
Albuminoid ,, ...	0·69	0·71	0·89	0·12	0·14	0·15
Nitrite ,, ...	nil	nil	nil	0·08	0·05	0·024
Nitrate ,, ...	nil	nil	nil	0·52	0·09	0·44
Oxygen absorbed from $KMnO_4$ in 4 hours ...	2·27	3·54	3·76	1·12	0·62	0·69
Chlorine	4·9	7·1	8·8	4·2	5·0	7·3

TABLE IV.

Average monthly analysis of sewage 1924–25 : *parts per 100,000.*

Date	Amm. nitrogen	Alb. nitrogen	Oxygen absorbed in 4 hours
August, 1924 ...	1·75	0·93	2·34
September... ...	1·3	0·61	2·74
October	1·05	0·45	2·70
November... ...	1·83	0·66	1·89
December... ...	2·5	0·46	2·2
February, 1925 ...	2·55	0·64	3·06
March	2·17	0·40	3·35
April	2·53	1·02	4·40
May	2·50	0·68	3·70
June	2·39	0·62	...

Chart II.
Results of
ANALYSES OF EFFLUENT



The results indicate that the treated sewage is rather weak, compared with the standards of the Royal Commission and other tests; the effluent has been of a high degree of purity. Tests were made to note the period of detention of sewage in the tanks, and the collection of sewage and effluent was made at such times as to yield comparable samples. The percentage purification effected in relation to flow may also be noted. The average monthly analyses of effluent for 1922–23 and those of sewage and effluent for 1924–25 are given in Chart II and Table IV. Comparing the analytical figures obtained for Indian sewages with those found in Western countries brings out the difference in character and strength of sewage due to the different habits of the respective populations.

TABLE V.

Analysis of different sewages: parts per 100,000.

Origin	Suspended solids	Amm. nitrogen	Alb. nitrogen	Oxygen absorbed in 4 hours	Chlorine
Indian					
Indian Institute of Science ...	9	2·44	0·71	3·54	7·1
Kurseong (hill station) ...	42·4	...	...	6·5	10·4
Dacca	12·5	5·0	0·24	3·6	9·6
Matunga (Bombay)	31·8	1·08	1·62	...	3·36
Sibpur (Calcutta)	25·6	0·17	0·02	7·1	17·00
Jamshedpur (Bihar)	...	1·88	1·98	11·26	3·5
Madras	...	3·30	1·26	7·6	17·5
English					
Saltley	57·7	4·06	1·52	26·34	18·9
Reading	34·2	10·4	1·48	8·8	10·1
Tinsley	27·3	3·38	0·62	5·4	...
Sheffield	77·4	5·09	1·22	13·93	...
Davyhulme (Manchester) ...	17·8	2·32	0·76	7·21	10·8
Withington (Manchester) ...	...	2·23	0·67	3·76	4·4
American					
Milwaukee	25·9	1·56	0·89	12·5	19·4

One of the interesting observations made while operating the plant was that chemical analysis of the effluent gave some index to the biology of the sludge. The sludge, in August, 1923, and March, 1925, was found to 'bulk', increasing abnormally in volume and settling slowly. Microscopic examination revealed huge masses of filaments and a chemical analysis of the effluent gave the following figures :—

Analysis of effluent : August, 1923 : parts per 100,000.

	1	2	3
Amm. Nitrogen	1·0	1·1	0·9
Alb. ,, ...	0·32	0·28	0·24
Nitrite ,, ...	Trace in 50 c.c.	Trace	Nil
Nitrate ,, ...	Nil	Nil	Nil
Total solids in 100 c.c. sludge	1·7 gm.	1·3 gm.	1·2 gm.

Analysis of sewage and effluent : March, 1925.

	Sewage				Effluent			
	1	2	3	4	1	2	3	4
Amm. nitrogen ...	2·2	2·1	2·5	1·87	1·98	1·05	1·8	1·08
Alb. ,, ...	0·42	0·48	0·44	0·16	0·26	0·24	0·11	0·12
Nitrite ,, ...	...	...	...	...	Nil	Trace	Trace	Nil
Nitrate ,, ...	...	...	...	...	Nil	Nil	Nil	Nil

It follows that purification in these circumstances was only 50 per cent. or less, due to the large masses of filament, much of which was suspended in the settlement tank and interfered with settlement.

Another point of importance noticed in varying the air-supply was that in spite of liberal aeration the dissolved oxygen-content of the final effluent never approached saturation, the highest figure being 0·3 parts per 100,000. Although Winkler's method indicated only very small quantities, in other respects the effluent was quite good. Incubation tests carried out on two different occasions with mixtures of effluent (10 per cent.) and aerated tap-water gave the following results.

Dissolved Oxygen in parts per 100,000.

Effluent	Aerated tap water	Effluent + Aerated tap water, c c.	
		0 hr.	12 hrs.
I ... nil	0·74	0·676	0 24
II ... nil	0·69	0·621	0·14

These showed that the effluent was not putrefactive, as in such a case all the dissolved oxygen would have been used up within 24 or 48 hours. The presence of dissolved oxygen even after 120 hours' incubation proved that the effluent was of high purity. It was therefore considered probable that the microbiological forms in the sludge might be responsible for the low percentage of oxygen in the final effluent. Some experiments were therefore conducted in the laboratory on the following lines. Equal volumes of sewage were collected from the various aeration chambers, mixed and the mixture divided between two narrow-mouthed graduated cylinders and allowed to settle for an hour. The supernatant liquid from one cylinder was siphoned into a third cylinder and aerated comparatively with complete sewage for varying periods, when the dissolved oxygen in the clear liquid after one hour of settlement was determined in the usual manner. After 24 hours' aeration the dissolved oxygen in parts per 100,000 was as follows :—

Complete sewage	0·06	0·17	0·13	0·12	0·11
Supernatant liquid	0·39	0·56	0·55	0·65	0·33

The results show that the dissolved oxygen in the supernatant liquid is many times more than that in the complete sewage containing the sludge. Microscopic examination showed that the sludge was teeming with life, and with increased aeration large numbers of ciliated organisms developed. The effluent was free from any such kind of life. It is therefore quite probable that the living population of the sludge was using up the major portion of the oxygen injected into the tanks.

A measure of the oxygen up-take by the organisms was, therefore, made as follows. A sample of tap-water was aerated until saturated with oxygen and gently shaken with sludge until intimately mixed; after settlement for an hour, the dissolved oxygen was estimated in the supernatant liquid. The residual sludge was again gently shaken with aerated tap water and the dissolved oxygen once more estimated.

This operation was repeated many times on successive days and the following are the average results expressed in parts per 100,000, the dissolved oxygen in saturated tap-water being 0·69.

1	2	3	4	5
0·03	0·07	0·06	0·09	0·09

The results show that there seems to be no limit to the amount of oxygen taken up within the period of the experiments conducted.

Biological observations.

Microbiology of the sludge.—The bacterial and non-bacterial micro-organisms in activated sludge are varied and numerous. The bacterial population included the following types:—Ammonifying, nitrifying, denitrifying and nitrogen-fixing. These were demonstrated by inoculating suitable media and observing the several changes characteristic of the respective types.

The non-bacterial life ranged from minute protozoa to worms, insect larvae and insects. The following were among the more important forms recognised:—

1. Protozoa.—*Amœba Paramoecium*, *Vorticella*, *Stylorichia*, *Leucophrys*, *Cœnomorpha*, *Actinophrys* and *Acineta*.

2. Trochelminthes.—*Brachionus*, *Philodina* and *Distyla*.

3. Vermes.—*Dero* and *Auginllilla*.

4. Arthropoda.—*Diptera*, *Chironomus* and *Culex*.

Microscopic study showed whether the sludge was in condition or not, the presence of ciliated organisms being evidence of ample aeration while flagellate-forms denoted defective air-supply. This is in accordance with the results obtained by Richards and Sawyer at Rothamsted (*J. Soc. Chem. Ind.*, 1922, **41**, 62 T). Another result of defective aeration was the rapid development of *Chironomus* larvae and the appearance of the dero type of worms; occasionally culicine larvae could also be found.

The development of *Chironomus* larvae was important, being associated with an improper circulation of sludge in the aeration tanks. It was observed on two occasions that owing to low air-supply a portion of the sludge settled in some of the tanks. The *Chironomus* larvae were found to live at this low oxygen level and collected all the sludge particles into small cylindrical masses which were buoyed up to the surface of the liquid. The life history of this insect was carefully investigated. The eggs under the microscope resemble

little strings of beads enclosed in a mucilaginous matrix attached to the walls of the tanks. The larvae emerge from the eggs in less than 48 hours, are blood-red in colour, 10–15 mm. long and segmented. They possess bristly projections at both ends, swim with a wriggling, lashing motion and live in tubes made of the sludge particles. Millions of these larvae may accumulate and collect a large proportion of the sludge, which either floats or becomes firmly attached to the walls. The larva developed into the pupa, the pupa into the imago and this into the chrysalis, the complete life cycle being repeated in the tanks. Only the larval stage of the above insect gives trouble, and the other stages of its life did not affect the sludge. Vigorous agitation in the early stages prevented the sludge from settling: the larvae then found it difficult to develop, and consequently no trouble arose. They were very susceptible to the action of kerosine and the briskly moving larvae were at once destroyed by this means, losing their red colour and rising to the surface.

Seasonal variations of the different types were remarkable. *Amœbæ*, *Stylonichia*, *Leucophrys* and *Nematodes* were invariably noticed in the sludge from October to March. *Paramoecia* and *Rotifera* were observed to be more numerous from July to October though they were found throughout the year. *Vorticella* and *Acineta* developed very rapidly with ample air-supply and appeared in all seasons. The *Chironomus* insect and the dero worms appeared chiefly from August to November, although now and then a few could be recognised at other times. With low air-supply and 'bulked' sludge culicine larvae could always be found.

Apart from the appearance of non-bacterial life in the sludge, a study of the relationship between the higher forms and the bacteria present was of profound interest. From continued observations under the microscope it is believed that a major proportion of these higher organisms used the bacteria in the sludge as their food supply. *Vorticella* and *Rotifer* are two examples in which it is easy to see that this process takes place. Both organisms are microscopic, and reveal cilia with which they direct the sludge particles, and probably countless bacteria also into their mouths. To study this relationship, work similar to that of Fairbrother and Renshaw (*J. Soc. Chem. Ind.*, 1922, **41**, 134 T) was conducted. The researches of Russell and others have shown that, in the case of soils, if partial sterilisation is effected either by heating to 100°, or by treatment with volatile antiseptics which are subsequently removed, there is a marked increase in the amount of oxygen absorbed by the micro-organisms of the soil (*J. Agric. Sci.*, 1907, **2**, 305). Goodey also has shown that soil protozoa exert a depressing effect on the soil bacteria (*Proc. Roy. Soc.*, 1917, **89 B**, 297). Fairbrother and Renshaw state that 'in the case of sewage

purification the method which is most economical, least noxious, most rapid and which gives highest fixed nitrogen content in the sediment is the activated sludge process. This process appears to be checked from time to time with the coincident growth of zoological masses and *Paramoecia* which use as food the bacteria causing the purification. Treatment of the tanks by this group of dyes (triphenylmethane) should result in elimination of the protozoa without disturbance to the bacteria'. In the present experiments therefore the sterilising agent consisted of Meldola's blue, or New blue R; Acridine yellow; Methylene blue.

Each dye solution was prepared by dissolving one gram in 100 c. c. of distilled water. To estimate the antiseptic action, a hanging drop preparation of the sludge was made on a cover-slip and examined under the microscope in a Botcher's chamber. The organisms were noted and an equal quantity of the stock dye solution added to produce a 1/200 dilution, and the suspension again examined under the microscope. If the organisms were dead, the dye solution was diluted and the effect tried as before with greater dilutions. The procedure adopted was exactly similar to that of the previous workers. The following results were obtained:—

Dye.	Dilution						
	1/200	1/500	1/1000	1/2000	1/5000	1/10000	1/20000
Methylene blue	All organisms except *Chironomus* and *Dero* are killed; these are stained.	...	Protozoa killed; Rotifers stained but unaffected.	Protozoa killed.	Protozoa alive.	Protozoa alive.	Protozoa alive.
Acridine yellow.	,,	...		Protozoa killed; Rotifers unaffected but stained.	Protozoa killed.	,,	,,
Meldola's blue.	All paralysed ...	*Chironomus* and *Dero* stained but not killed.	,,	,,	,,	Protozoa killed.	,,

The highest dilution of each dye effective in killing the protozoal forms was therefore:—Methylene blue, 1/2000; Acridine yellow 1/5000; Meldola's blue, 1/10000. Quantities of the sludges after

treatment with dyes were then plated out on nutrient agar in Petri-dishes, dilutions being made in the usual manner, undyed controls being kept. The following counts were obtained :—

TABLE VI.

Influence of protozoa on bacterial numbers (1000's).

A		B		C	
Sludge and Meldola's blue	Sludge only	Sludge and methylene blue	Sludge only	Sludge and Acridine yellow	Sludge only
25	3	5	3	10	25
11	5	6	5	6	2
140	100	11	3	200	80
9	3	70	25	50	8
23	65	43	65	60	20
200	25	57	25	...	...
70	37	11	3	...	...
45	30	...	...	...	...
33	50	...	...	...	...
40	1,500	...	...	...	...
17	12	...	...	...	...
100	470	...	...	...	...
750	1,500	...	...	...	...

The above table clearly shows that when protozoa are inhibited, bacteria increase. In Nos. 9, 12 and 13, however. the total counts in the sludge without dye were the greater; these were all 'bulked' sludges, containing masses of the filamentous organism with few protozoa. Richards and Sawyer have obtained similar results (*J. Soc. Chem. Ind.*, 1922, **41**, 627) working with volatile antiseptics.

Experiments were made to ascertain also whether the absence of the protozoa accelerates nitrification. In three Erlenmeyer flasks of 100 c. c. capacity 50 c. c. of Winogradski's medium containing potassium hydrogen sulphate, magnesium sulphate and sodium chloride with a trace of calcium chloride were introduced, and sterilised in an autoclave at 10 lbs. pressure for 15 minutes. One gram of sterilised magnesium carbonate and 2 c. c. of a two per cent. solution of

ammonium sulphate were added to each ; one was kept as a control, the second inoculated with a small quantity of the treated sludge, while the third flask was inoculated with untreated sludge. All were incubated at 37° and examined for nitrites and nitrates once in two days. Nitrification proceeded so rapidly in the second and third flasks that within a week 0·1 part per 100,000 of nitrates could be estimated in each, while the control did not show any trace of nitrite or nitrate ; after three weeks only traces of ammonia were present. No appreciable difference in action of the treated and untreated sludges could be noticed. The experiments showed that inhibition of the protozoa did not induce more rapid nitrification.

Bulking.—That a low air-supply to activated sludge tanks also affects the biology of the sludge was shown by the phenomenon of 'bulking' already described. The volume of sludge then found in the aeration tanks suddenly becomes abnormal and increases with considerable rapidity during several days. The sludge also changed in physical character, was slow in settling, with less than 50 per cent. purification and the effluent contained a large quantity of suspended solids.

A microscopic examination revealed the immediate cause of bulking to be large masses of filamentous growths while higher forms of life were rare (nil in 0·01 c. c.). Sludge in the aeration tanks was measured by collecting equal samples, allowing settlement for an hour and reading the sludge level. Generally speaking, the amount built up in a day ranged from 2 to 5 per cent., but during the bulking period the percentage of sludge increased from 25 to 40 per cent. in two days and subsequently to 60 per cent., this percentage being maintained for a week. The sludge was grey and did not resemble a normal, healthy sludge. When allowed to settle, there appeared a white slimy mass above the sludge proper. The latter never settled quickly and the supernatant liquid always had in suspension some white flecks which appeared in the effluent also.

The filaments were straight rods of varying lengths enclosed in a membranous sheath. A culture of the organism was made on nutrient agar as well as on several sugar media. The multiplication was very vigorous and good growths were obtained within 18 hours. The organism is gram-positive, does not produce acid or gas and does not thrive in even slightly acid media. A drop of dilute acid added to the effluent flocculates these filaments, which appear to grow better in liquid cultures than on solid media. In the former case the sheath is preserved, while in the latter it is lost and the thread-like form disappears. The filaments on two occasions were found to be the cause of bulking, while protozoa, which have been stated to be the

cause, were found throughout the year with low or high air-supply without giving any trouble.

The filaments are probably *Sphærotilus natans* or a closely related organism. *Sphærotilus natans* has been described as a sewage organism by several workers, but although its various characteristics have been described, its functions do not seem to have been fully studied. According to Ohlmuller and Spitta (*Wasser und Abwasser*, 180) it is found in shallow, flowing water in the form of whitish flakes and skin-like incrustations. Its appearance is favoured by sewage or cellulose factory wastes and it is found in sugar refineries and brewery effluents. It consists of a series of cells enclosed in a sheath and forms in this manner thin threads of about 2μ diameter. *Sphærotilus* is found everywhere and is the most characteristic of drainage organisms. Similar descriptions are given by many other workers, e.g., Thresh, Bergey, David Ellis and Mrs. Mumford.

Whenever the sludge bulked, the only remedy was to reduce the flow of sewage and increase the supply of air for a prolonged period when the filaments rapidly broke up and were thus destroyed (O'Shaughnessy, *J. Soc. Chem. Ind.*, 1923, **42**, 359T).

Experiments on de-watering of activated sludge.—One of the difficulties encountered in this method of sewage treatment has been the disposal of the surplus sludge. Where large areas of land are available, no difficulty arises in the disposal; otherwise, a method of de-watering has to be adopted. Activated sludge retains water so tenaciously that de-watering presents a real problem which has not yet been solved; this remains the one weak point of the method, which in other respects has given most excellent results.

The volume of sludge pumped from the plant in question is between 3,000 and 4,000 gallons per week. The water-content is normally 97 to 97.5 per cent., but rose to 99 per cent. when the sludge bulked. All is pumped to a compost-pit where the sludge is de-watered and fermented to a valuable fertiliser.

De-watering experiments on a laboratory scale were conducted on the lines of Wilson and Heisig (*J. Ind. Eng. Chem.*, 1921, **13**, 406). Definite volumes of sludge, say, 100 c.c., were treated with varying amounts of N/2 sulphuric acid or aluminium sulphate solution, the mixture being then thoroughly stirred, poured on to Buchner funnels and filtered by suction through paper. The time taken for the sludge to filter, until free from visible moisture, was noted by a stop-watch and the P_H of the filtrate determined colorimetrically; the filtered solids were weighed at once and after drying at 105°. The results are recorded below.

TABLE VII.

Experiments with N/2 Sulphuric Acid (100 c.c. of sludge).

Acid c.c.	Time for filtration		Filtrate P_H	Percentage moisture in filter-cake
	Mins.	Secs.		
0	10	...	7·2	30·5
1	9	51	6·6	30·0
2	8	35	5·8	29·0
4	6	8	5·2	26·5
5	4	21	3·6	21·5
10	1	30	2·6	16·7
15	2	...	1·6	22·0

TABLE VIII.

Experiments with N aluminium sulphate (100 c.c. of sludge).

Alum c.c.	Time for filtration		Filtrate P_H	Percentage moisture in filter-cake
	Mins.	Secs.		
0	10	...	7·2	30·5
0·5	1	35	7	29·5
1	0	52	6·2	29·0
2	0	33	5·2	23·7
5	0	40	4·7	26·5
10	1	...	3·8	24·5

From this it is clear that in the case of sulphuric acid filtration was quickest at a P_H of 2·6, when the moisture was also the lowest. Either above or below this range of P_H, the moisture-content increased as well as the time taken for filtration. Addition of acid produced bubbles of gas and the colour changed from brown to yellow. Addition of alum produced a porous filter-cake which could not maintain a vacuum of more than 30 c.m., and a sharp minimum was attained at P_H 5·2. It produced a turbidity in the supernatant liquid, otherwise

there was no change. The moist filter-cake in neither case produced any smell when exposed for 3 days.

De-watering was also tried on a small scale by centrifuging 50 c.c. at a time at 2,500 to 4,000 r.p.m. for 10 minutes. The moisture-content of the resultant sludge was still 40·8 per cent. however. Experiments in drying on sand and turf beds have also given fairly satisfactory results. Sludge on a turf bed dried more slowly than on a sand bed, but while in the latter case the sludge became incorporated with the sand, the former gave a cake yielding to the spade. The dried products, even after storage for many months, gave no offensive smell. On the whole, treatment with alum appears the most promising line of attack. Analyses of dried sludge are given in table IX.

TABLE IX.

Analysis of air-dried activated sludge (per cent.).

	1	2	3	4	5	6	7	8
Moisture ...	7·94	8·12	8·1	8·14	...	...	...	..
Organic matter ...	66·00	69·4	64·4	70·2	69·6	67·0	74·41	75·51
Total nitrogen ...	3·33	4·0	4·69	5·0	4·94	4·95	5·01	5·02
Phosphate as P_2O_5 ...	...	...	...	2·3	...	...	2·89	3·24
Potash as K_2O ...	...	...	...	0·5	...	...	...	0·88

Elasticity of the process.—Although the plant was intended to treat 6,000 gallons of sewage per day, actual experience has shown that it is able to work on either side of this figure to a considerable extent. Laboratory experiments have also shown that activated sludge can become acclimatised to treat trade-effluents with success. The writer was able to secure quantities of creamery and tannery-wastes, and treatment of these liquors by activated sludge was eminently successful. In both cases the waste liquors were foul, but, by inoculating them with 20 per cent. activated sludge and aerating for two days, an odourless, innocuous liquid was obtained, colourless from creamery-waste and still coloured from tannery-waste. The process seems to be very sensitive to acid, but if this is neutralised the sludge begins to function. Sludges were treated with sulphuric acid and aerated for a number of days without producing any effect, but immediately after neutralisation the activity was restored. The nitrifying organisms seem to function best between P_H 7 and 8, although they thrive well between 5·8 and 8.

Economics of the process.

The Capital cost of the plant was as follows :—

	Rs.
Constructional work	4,969
Equipment from Messrs. Activated Sludge, Ltd.	5,064
Reavell Compressor	400
Scott ,, (approx.)	700
3 h.p. Motor and cable	592
5 h.p. Motor	453
3 Zinc sheds for motor and compressor	397
Grates, plate covers, etc.	88
Total	12,663

Maintenance cost per annum :—

	Rs.
Interest at 6 per cent. per annum	760
Depreciation at 12 per cent. per annum	258
Power consumption, 62 units per day, at 6 pies per unit	707
Chemist in charge	840
Watchmen	372
Lubricating oil, cotton waste, etc.	66
Total	3,003

On the above basis the capital cost is Rs. 33-12-0 per head of population (375), or Rs. 5-10-0 per 1,000 gallons treated, assuming 6,000 gallons per day, yearly. The maintenance costs are Rs. 8 per head per annum, or Rs. 1-6-0 per 1,000 gallons treated. For a larger plant these rates would be very materially reduced.

Summary and conclusions.

The plant has been in operation continuously for a period of more than four years except for a few days from time to time owing to failure of current and other circumstances. The plant had to be re-started on two occasions when the rate at which nitrification was established and the building up of sludge have been much more rapid than on the previous occasion. A plant of this type appears to possess the following advantages :—

1. The sewage may be purified to any desired extent,

2. The space occupied by the plant is only about one-third that required by a closed septic tank and bacteria-bed, or any other form of sewage-treatment plant.

3. The treatment yields a sludge which contains a high percentage of nitrogen and is therefore valuable as a fertiliser.

4. Maintenance costs are low, as an ordinary workman trained for the purpose can control the plant with occasional expert advice.

Low air-supply tends to produce bulking and the development of numerous insect larvae and worms. Ample aeration obviates the above troubles and restores order. No trouble with the diffusers has arisen except occasionally when a large quantity of silt has been deposited, removed by steaming.

The average effluent has been quite satisfactory; although nitrates were present in large quantities, the dissolved oxygen has always been very low.

The biological forms in the tanks have shown a seasonal variation and the presence of filamentous growths always contributed to an unhealthy and bulked sludge. There seems to be a relation between the protozoal and the bacterial numbers, the former tending to keep down the latter.

Though no necessity for de-watering the sludge has arisen, the experiments show that after suitable treatment the sludge can be rapidly filtered, yielding a filter-cake with a comparatively low moisture-content.

In conclusion, I am glad to acknowledge my indebtedness to Professors Gilbert J. Fowler and R. V. Norris for their keen interest in the work and for their continued guidance throughout the course of this investigation.

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CONTRIBUTIONS TO THE STUDY OF SPIKE-DISEASE OF SANDAL (SANTALUM ALBUM, LINN.).

Part VI.—Nitrogen Metabolism in Healthy and Spiked Sandal Leaves.

By N. Narasimhamurthy and M. Sreenivasaya.

The nitrogen metabolism of sandal is one of fundamental interest, both physiological and pathological. Pot-culture experiments (unpublished) have shown the indispensability of providing hosts, more especially leguminous, for the healthy and vigorous growth of sandal. Without a host, sandal continues to have a struggling existence and one of the essential limiting factors appears to be nitrogen. When a suitable host is given to the starving sandal, the pale yellow leaves characteristic of nitrogen deficiency gradually become deep green, and the sandal plant as a whole begins to show visible growth.

Analyses have proved that with the onset of spike the total nitrogen of diseased tissues, whether leaves, stem or root is generally higher than that of the corresponding tissues of healthy sandal. We have seen that the vigorously growing sandal draws a large proportion of nitrogen from its host plant and it is curious to find that there is a further increase in the total nitrogen in the diseased state.

Two problems arise in this connection :—(*a*) The ultimate source of the extra nitrogen accumulating in pathological sandal tissues, and (*b*) The nature of the nitrogenous constituents in the diseased tissues. The present communication is a comparative study of the various forms of nitrogen in the healthy and the diseased leaves of sandal.

MATERIAL.

The leaves employed in this investigation were collected from two areas, Uttarahalli and Ragihalli, the former lying 6 miles south-west of Bangalore and the latter lying 13 miles south of Bangalore : the samples were collected at about 9 a.m. The characteristic external symptoms were the guiding factors in the collection of the diseased and healthy samples. The healthy leaves measured on an average 6·1 cm. in length and 2·6 cm. in breadth, the diseased leaves being 2·4 cm. long and 0·5 cm. broad. The samples were conveyed

in stoppered bottles by motor to the laboratory immediately after collection. The leaves were separated from twigs, spread on trays, and rapidly dried in a vacuum oven under reduced pressure, over fused calcium chloride between 35° and 40°. These precautions were taken to minimise as far as possible the biochemical changes *in vitro* affecting the various nitrogenous constituents. The samples, after 48 hours' drying, were ground in a mortar, passed through a 30-mesh sieve and preserved in covered jars.

Moisture was estimated by drying in a steam oven at 95° to constant weight. At each weighing of a sample for analysis, a re-determination of moisture was necessary, the leaves having been highly desiccated during the above process of drying; these moisture-values have been employed in calculating results on the moisture-free basis.

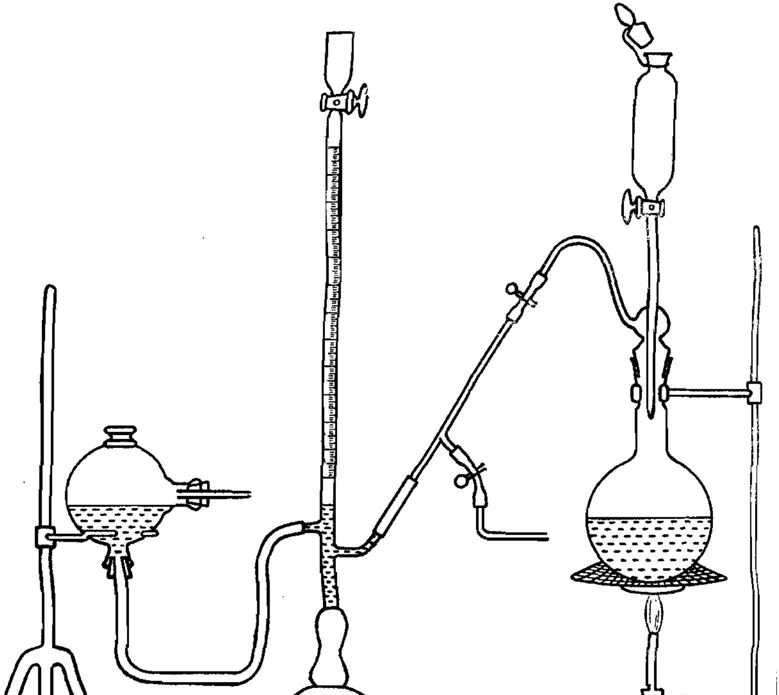
The two sets of samples employed for the investigation were so chosen as to represent extreme types. The Ragihalli samples represent the usual type, the healthy leaves possessing a lower nitrogen value, while in the other set of samples from the Uttarahalli area, the healthy leaves have a higher percentage of total nitrogen than the spiked leaves.

METHODS.

Total Nitrogen.—Total nitrogen was determined by Gunning's modification of Kjeldhal's method to include nitrate nitrogen (A.O.A.C., Methods of Analysis). The data represented in Table I are the averages of two or more individual analyses.

Protein Nitrogen.—Protein nitrogen was estimated by a method proposed by Ritthausen and Heinrich (*Die Eiweisskörper der Getreidearten, Hulsenfrüchte und Olsamen*, p. 252; Bonn, 1872) and perfected by Stutzer (*J. Land.*, 1880, **28**, 103). The leaf powder (1 g.) was treated with 100 c.c. of water, heated to boiling and kept on the steam bath for about 10 minutes; 2 c.c. of a saturated potassium-alum solution was added followed by 30 c.c. of Stutzer's solution (corresponding to 0·45 g. of copper hydroxide), and the whole was well stirred. On cooling, the insoluble residue was filtered, washed with water, and the nitrogen estimated according to Kjeldahl's method. Nitrogen in the filtrate was also estimated as a check. The average values are recorded in Table IV.

Nitrate Nitrogen.—Nitrate nitrogen in the sandal leaves was estimated according to the method of Schulze and Tiemann (Abderhalden's *Biochemische Arbeitsmethoden*, 1912, 6312). The leaf powder (10 g.) was repeatedly extracted with 80 per cent. alcohol, of which



about 400 c.c. was used in all. The extract was made alkaline with lime and evaporated to dryness in vacuum at 40–45°, the residue being dissolved in hot water, transferred to a 1000 c.c. measuring flask, treated with lead acetate and made up to 1000 c.c. After the precipitate had settled, the solution was decanted into a filter and 200 c.c. portions of the perfectly clear filtrate used each time for the estimation of nitrate nitrogen. The apparatus (see figure) included a 250 c.c. ground stoppered wash bottle in which the long tube was cut about 6 cm. below the neck and drawn to a capillary opening, a 50 c.c. separating funnel being fused on to the other end. The delivery tube was connected to a nitrometer through a T-piece by means of thick-walled rubber tubing; pinchcocks were used at the places indicated. The joint at the neck of the reaction flask escapes attack by the concentrated hydrochloric acid, and was ground with emery to make it perfectly air-tight; the length of the ground contact being about 5 cm., the pressure developed by steam during manipulation does not push up the stopper. The nitrometer was filled with 50 per cent. caustic alkali. The solution to be analysed for nitrate was concentrated in the flask to about 25 c.c., air in the apparatus being thus displaced by steam. After cooling, 25 c.c. of ferrous chloride solution (prepared by treating 400 g. of iron nails with a litre of concentrated hydrochloric acid) followed by 25 c.c. of concentrated hydrochloric acid was sucked through the separating funnel. The liberated nitric oxide was transferred to the nitrometer by alternate boiling and cooling of the mixture in the flask. The volume of nitric oxide collected in the nitrometer was measured at room temperature and pressure. Blank determinations were made with reagents alone. The blank value which came up to 0·05 c.c. after correcting the volume to N.T.P. was subtracted from the N.T.P. value of the nitrometer reading, and this figure was taken to represent the actual volume of nitric oxide evolved. The nitrate content in 0·2 per cent. potassium nitrate solution was also estimated, the results agreeing within 1 per cent. of the theoretical value.

The Ragihalli samples contained very small quantities of nitrate nitrogen, and accordingly the colorimetric method of estimation was used. The clear filtrate prepared as above (100 c.c.) was evaporated to dryness in a porcelain dish, treated with 1 c.c. of phenoldisulphonic acid, leached with water and then transferred to a Nessler's tube; 1 c.c. of 4 *N* potassium hydroxide was then added, and the volume made up to 50 c.c. The yellow tint was compared with that produced by a standard solution of potassium nitrate containing 0·01 mg. of nitrate nitrogen per c.c. of the solution. The data represented in Table IV (series 3) are the mean of more than one individual determination.

Nitrite Nitrogen.—The clear solution prepared for the nitrate determination (50 c.c.) was placed in a Nessler's tube to which was

added 1 c.c. of 1 per cent. sulphanilic acid and 1 c.c. of α-napthylamine acetate. The volume was made up to 100 c.c. and the pink colour compared with that produced by a standard solution of sodium nitrite containing 0·01 mg. of nitrite nitrogen per c.c. The results are represented in Table IV (series 4).

Ammonia Nitrogen.—Ammonia nitrogen in the sandal leaves was estimated according to Grafe's method (Grafe and Erich, *Zeits. Physiol. Chem.*, 1906, **48**, 300). The leaf powder (10 g.) was placed in a 500 c.c. Claisen flask and washed down with 25 c.c. of ammonia-free distilled water, 25 c.c. of saturated sodium chloride solution and 12·5 c.c. of alcohol. The flask was then connected to a one litre filter-flask containing 40 c.c. of 0·05 *N* sulphuric acid to absorb the ammonia, and cooled in ice: saturated sodium carbonate solution (12·5 c.c.) was then added and the mixture distilled under reduced pressure (15 mm.) on a water bath at 42–44°. After 6 hours of distillation, the acid in the filter flask was back titrated against standard alkali. The ammonia found by this method was taken to represent the free ammonia in the aqueous leaf extract, and the values are represented in Table IV (series 6).

Water-soluble Nitrogen.—The determination of the distribution of water-soluble nitrogen was made according to Hausmann's method (Hausmann and Walther, *Zeits. Physiol. Chem.*, 1899, **27**, 95; Osborne and Harris, *J. Amer. Chem. Soc.*, 1903, **25**, 323) and as applied to the spinach materials by Jodidi and co-workers (Jodidi, Kellogg and True, *J. Agric. Research*, 1918, **15**, 385). The method of extracting the water-soluble nitrogen as well as the methods of determining the various nitrogenous constituents (amide, humin, basic, etc.,) were strictly those detailed in the above paper, and a description of the methods will therefore not be attempted here. The amino-nitrogen in the water-soluble extract, however, as well as the mono-amino-nitrogen and the peptide nitrogen were determined according to the method of Van Slyke (*J. Biol. Chem.*, 1911, **9**, 185 and 1912, **12**, 275).

The results obtained by these methods are summarised in Table II, and are expressed as percentages on the weight of the moisture-free material. Table III expresses the same results as percentages of the total water-soluble nitrogen. Column I (Table II) gives the total water-soluble nitrogen as obtained by the digestion of 20 c.c. of the water-extract with sulphuric acid according to the method of Kjeldahl; and therefore the value of the total water-soluble nitrogen is possibly less by the amount of nitrate and nitrite nitrogen. Column 6 (Table II) gives the results of the mono-amino-nitrogen determinations made

according to the method of Van Slyke on 10 c.c. aliquots of the 100 c.c. of non-basic extract. The filtrate, after removing the phosphotungstic acid precipitate of basic nitrogen, was freed from excess of phosphotungstic acid by means of baryta, excess of which was removed by means of carbon dioxide. The filtrate was then concentrated, made up to 100 c.c. and was characterised as the non-basic extract. Column 7 (Table II) gives the difference between the total non-basic nitrogen (as determined by the Kjeldahl's method on 20 c.c. portions of the non-basic extract) and the mono-amino-nitrogen in column 6: and this is characterised as the non-amino-nitrogen in the non-basic extract. The difference between the amino-nitrogen found in 10 c.c. of the 100 c.c. of peptide extract and in a similar aliquot of the water-soluble extract gives the peptide nitrogen; the values are recorded in column 8 (Table II). The peptide extract was obtained by adding concentrated hydrochloric acid (20 per cent.) to 100 c.c. of the water extract and then hydrolysing the mixture for 8 hours. After removing the amide and humin nitrogen, the filtrate after concentration was made up to 100 c.c. and represented the peptide extract.

TABLE I.

Percentage total nitrogen in the sandal leaves on the moisture-free basis.

No.	Sample	Healthy	Diseased
1	Ragihalli, 15-8-28 ...	1·733	2·052
2	Uttarahalli, 12-9-28 ...	3·318	3·030
3	Ragihalli, 15-2-29 ...	1·416	2·261

DISCUSSION OF RESULTS.

Table I shows that in the three sets of samples analysed, the total nitrogen of diseased leaves from Ragihalli is higher than that of the healthy leaves from the same area. The healthy leaves from the Uttarahalli area have a higher percentage of total nitrogen, obviously due to the association of a richly leguminous host with the sandal from which the samples were derived. This is an observation which has been confirmed by numerous analyses and is one of vital importance in the choice of samples for analysis. Uttarahalli samples always show a higher nitrogen content than the Ragihalli samples, which, as already shown, is attributable to the fact that, in the Uttarahalli area,

TABLE II.

Nitrogen distribution in the water-soluble portion expressed in percentages of the oven-dried sandal leaves (on the moisture-free basis).

No.	Material and date of collection	Treatment HCl per cent.	1 Total water-sol. N	2 Ammonia N	3 Amide N	4 Humin N	5 Basic N	6 Mono-amino N	7 Non-amino in non-basic extract	8 Peptide N
1	Uttarahalli, 12-9-28 Healthy ...	20 for ½ hr.	0·935	0·013	0·073	0·121	0·236	0·163	0·467	...
2	,, ,, ,, ...	4 for 2 hrs.	0·935	0·013	?	0·118	0·214	0·110	0·501	...
3	,, ,, ,, ...	20 for 8 hrs.	0·935	0·013	0·066	0·114	...	...	...	0·008
4	,, ,, Diseased ...	20 for ½ hr.	1·167	0·015	0·033	0·160	0·291	0·158	0·520	...
5	,, ,, ,, ...	4 for 2 hrs.	1·167	0·015	0·030	0·167	0·306	0·111	0·586	...
6	,, ,, ,, ...	20 for 8 hrs.	1·167	0·015	0·043	0·167	...	...	...	0·000
7	Ragihalli, 15-2-29 Healthy ...	20 for ½ hr.	0·460	0·005	0·022	0·211	0·048	0·043	0·124	...
8	,, ,, ,, ...	4 for 2 hrs.	0·460	0·005	0·020	0·166	0·046	0·042	0·177	...
9	,, ,, ,, ...	20 for 8 hrs.	0·460	0·005	0·021	0·209	...	...	...	0·002
10	,, ,, Diseased ...	20 for ½ hr.	0·988	0·009	0·057	0·200	0·172	0·044	0·521	...
11	,, ,, ,, ...	4 for 2 hrs.	0·988	0·009	0·055	0·177	0·179	0·034	0·593	...
12	,, ,, ,, ...	20 for 8 hrs.	0·988	0·009	0·056	0·209	...	...	...	0·000

TABLE III.

Distribution of water-soluble nitrogen expressed in percentage of the total soluble nitrogen.

No.	Material and date of collection			Treatment : HCl per cent.	1 Total water-sol. N	2 Ammonia N	3 Amide N	4 Humin N	5 Basic N	6 Mono-amino N	7 Non-amino in non-basic extract	8 Peptide N
1	Uttarahalli, 12–9–28.		Healthy ...	20 for ½ hr.	100	1·390	7·81	12·9	25·2	17·4	49·9	...
2	,,	,,	,,	4 for 2 hrs.	100	1·390	?	12·6	22·9	11·8	53·6	...
3	,,	,,	,,	20 for 8 hrs.	100	1·390	7·06	12·2	...	...	...	0·856
4	,,	,,	Diseased.	20 for ½ hr.	100	1·290	2·83	13·7	24·9	13·5	44·6	...
5	,,	,,	,,	4 for 2 hrs.	100	1·290	2·57	14·3	26·2	9·51	50·2	...
6	,,	,,	,,	20 for 8 hrs.	100	1·290	3·68	14·3	...	..	...	0 000
7	Ragihalli, 15–2–29.		Healthy ...	20 for ½ hr.	100	1·090	4·78	45·9	10·4	9·35	27·0	...
8	,,	,,	,,	4 for 2 hrs.	100	1·090	4·35	36·1	10·0	9·13	38·5	...
0	,,	,,	,,	20 for 8 hrs.	100	1·090	4·57	45·4	...	...	...	0·435
10	,,	,,	Diseased.	20 for ½ hr.	100	0·911	5·77	20·2	17·4	4·45	52·7	...
11	,,	,,	,,	4 for 2 hrs.	100	0·911	5·57	17·9	18·1	3·44	60·0	...
12	,,	,,	,,	20 for 8 hrs.	100	0·911	5·67	21·2	...	...	...	0·000

TABLE IV.

Nitrogen distribution in the sandal leaves.

No.	Form of nitrogen	Uttarahalli Samples, 12-9-28				Ragihalli Samples, 15-2-1929			
		Percentage of the oven dry material (on the moisture-free basis)		Percentage of total nitrogen		Percentage of the oven dry material (on the moisture-free basis)		Percentage of total nitrogen	
		Healthy	Diseased	Healthy	Diseased	Healthy	Diseased	Healthy	Diseased
1	Total ...	3·318	3·030	100	100	1·416	2·261	100	100
2	Protein ...	2·038	1·690	61·42	55·78	0·981	1·235	69·28	55·89
3	Nitrate ...	0·234	0·056	7·053	1·848	0·004	0·001	0·283	0·044
4	Nitrite ...	0·003	0·001	0·090	0·035	0·0001	0·000	0·007	0·000
5	Total water-soluble ...	0·935	1·167	28·18	38·52	0·460	0·988	32·49	43·70
6	Ammonia ...	0·013	0·015	0·392	0·495	0·005	0·009	0·353	0·358
7	Amide ...	0·070	0·032	2·11	1·056	0·021	0·056	1·483	2 477
8	Total amino ...	0·275	0·377	8·288	12·44	0·076	0·143	5·367	6·325
9	Humin ...	0·118	0·165	3·556	5·446	0·210	0·205	14·830	9·067
10	Basic ...	0·225	0·299	6·781	9·868	0·047	0·176	3·319	7·784
11	Peptide ...	0·008	0·000	0·241	0·000	0·002	0·000	0·141	0·000

sandals are to be found in the midst of luxuriant and leguminous hosts aided by cultivation. The Ragihalli sandals do not have these advantages, the soil is barren and rocky, leguminous hosts are rare, and there is no cultivation. It is pertinent to reiterate that the composition of the parasitic sandal will vary with the nature of the associated host-plants and that the results of one area cannot be compared with those of another whose ecological environment is widely different.

However, the higher nitrogen-content generally found in the diseased tissues of sandal is in strange contrast with the usually lower percentages of nitrogen found in the diseased tissues of spinach blight, tobacco mosaic, curly top of sugar-beet, etc. The accumulation of the extra nitrogen in the leaves with the onset of spike disease is possibly due to one of the following factors:—(1) Abnormal distribution of the constituents due to unbalanced translocation, (2) Selective absorption of nitrogenous components from the host or soil or both by the sandal in its pathological state, and (3) The infective principle responsible for causing the disease being possibly a nitrogen-fixer.

An examination of Table IV (series 2) reveals that the protein content (as determined by Stutzer's method) is higher in the healthy than in the diseased leaves for both the areas when calculated as percentage of the total nitrogen. There seems to be a remarkable tendency in the plant to maintain an equilibrium between the total nitrogen and the protein nitrogen, an increase in the total nitrogen being followed by a corresponding increase in the protein nitrogen. In the diseased leaves also, the same phenomenon is noticeable, although the ratio of protein nitrogen to total nitrogen is less than that in the case of the healthy plants, due probably to the degradation of the proteins. The higher proteoclastic activity exhibited by the diseased tissues and the higher content of the total water-soluble nitrogen also testify to the above assumption. This higher content of the water-soluble nitrogen and the ratio of the protein to the water-soluble nitrogen given below, may well be marked as characteristics of the disease.

Ratio of the protein to the water-soluble nitrogen of leaves.

		Healthy	Spiked
Uttarahalli	...	2·18	1·44
Ragihalli	...	2·13	1·19

Reviewing the distribution of water-soluble nitrogen, we find that the humin nitrogen calculated as percentage of the dry-weight material (Table III, column 4) is not significant.

The nitrate-content of spiked leaves is strikingly low and possibly points to the poor absorption of the nitrates from the host or the soil, in view of the fact that in the diseased condition sandal loses its haustorial connections and root-ends.

Basic nitrogen is present in greater quantities in the diseased than in healthy samples for both areas, when calculated both in terms of water-soluble nitrogen and of moisture-free material. This seems to be a distinguishing characteristic of the disease.

Examination of Table II (column 6) shows that the value of mono-amino-nitrogen fluctuates according to the concentration of hydrochloric acid used in hydrolysing the water-soluble extract. The solution treated with 4 per cent. hydrochloric acid for 2 hours contains always a lower percentage of mono-amino-nitrogen than that treated with 20 per cent. hydrochloric acid for ½ hour. This fact goes hand in hand with the observation that when the solution is treated with 4 per cent. hydrochloric acid for 2 hours there is present in the non-basic portion a greater quantity of non-amino-nitrogen than when the solution is treated with 20 per cent. hydrochloric acid for ½ hour. The values represented in Table II (column 6) cannot, however, be taken to represent the true amount of mono-amino-nitrogen in the samples, because it has been proved that a part at least of the humin nitrogen is formed at the expense of the mono-amino-acids while boiling with hydrochloric acid (Hart and Bentley, *J. Biol. Chem.*, 1915, **22**, 477; Roxas, *J. Biol. Chem.*, 1916, **27**, 71).

An examination of Table IV reveals that the total amino-nitrogen is greater in the spiked than in the diseased plant for both areas, whether expressed as percentage of the dry-weight material or as percentage of the total nitrogen. This increase in amino-nitrogen seems to be another characteristic of the disease, indicating proteoclastic degradation of proteins in the leaf.

SUMMARY.

1. The total nitrogen in spiked leaves is generally greater than in healthy leaves, and the total nitrogen-content varies with the locality.

2. As physiological characteristics of the disease there may be put forward an increase in (1) total water-soluble nitrogen, (2) basic nitrogen, and (3) total amino-nitrogen, and a decrease in the nitrate nitrogen in the diseased leaves, both in reference to the dry-weight of the leaves and in reference to the total nitrogen.

3. The lower content of protein nitrogen (with reference to the total nitrogen) taken along with the lower content of the polypeptide nitrogen and increase in amino- and water-soluble nitrogen seems strongly to indicate a proteoclastic degradation of the proteins proceeding in the diseased leaves.

4. A detailed investigation of the basic constituents has been undertaken and the work will be extended to an examination of the stems and roots of sandal.

In conclusion, we wish to express our grateful thanks to Professor Roland V. Norris for his many helpful criticisms and the keen interest he has evinced throughout this work.

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LENGTHENED *ORTHO*-DI-DERIVATIVES OF BENZENE AND THEIR RING-CLOSURE: FORMATION OF POLYMEMBERED HETEROCYCLIC COMPOUNDS FROM SUBSTITUTED PHENYLENE-DICARBAMIDES.[1]

By Tejendra Nath Ghosh and Praphulla Chandra Guha.

The ring-closing action of hydrochloric acid, sodium hydroxide and acetic anhydride upon hydrazodicarbamides, mono- and dithioamides has been studied in some detail and a large number of thiodiazole and triazole derivatives have been obtained (Freund, *Ber.*, 1895, **28**, 946; 1896, **29**, 2506; Arndt and co-workers, *Ber.*, 1921, **54**, 2089; 1922, **55**, 341; Fromm, *Annalen*, 1923, **433**, 1; *Monatsh.*, 1924, **44**, 298; *Ber.*, 1923, **56**, 1370; Guha, *J. Amer. Chem. Soc.*, 1922, **44**, 1502; 1923, **45**, 1036; 1925 **47**, 387). It was thought worth while to extend the field of such dicarbamides so as to comprise the corresponding —CO·NHR and —CS·NHR derivatives of aromatic and aliphatic 1 : 2-diamines. Phenylene- and ethylenediamines, it is well known, form dithiocarbamides with ammonium sulphocyanide which readily form phenylene- or ethylenethioureas. The following mixed and simple disubstituted dicarbamides as also the mono- and dithiocarbamides[2] have now been prepared from *o*-phenylene- or *o*-tolylenediamine.

$C_6H_4(NH{\cdot}CS{\cdot}NHPh)_2$ (I)

$C_6H_4(NH{\cdot}CS{\cdot}NH{\cdot}C_6H_4{\cdot}CH_3)_2$ (*o*- and *p*-) (II, III)

$C_6H_4(NH{\cdot}CS{\cdot}NH{\cdot}C_6H_3(CH_3)_2)_2$ (IV)

$CH_3{\cdot}C_6H_3(NH{\cdot}CS{\cdot}NHPh)_2$ (V)

$C_6H_4(NH{\cdot}CS{\cdot}NH{\cdot}CH_3)_2$ (VI)

$C_6H_4(NH{\cdot}CS{\cdot}NH{\cdot}C_3H_5)_2$ (VII)

$$C_6H_4\begin{cases}NH{-}CS{-}NHPh\\ NH{-}CS{-}NH{\cdot}C_3H_5\end{cases}$$ (VIII)

[1] Reprinted from the *Journal of the Indian Chemical Society*, 1929, **6**, 181.

[2] Compounds I, VII, XV are known (*Annalen*, 1885, 228, 200, *Ber.*, 1883, 16, 592) but no unsymmetrically substituted mono- or dithiodicarbamide is known.

$$C_6H_4\left<\begin{array}{l}NH—CS—NHPh\\NH—CS—NH—C_6H_4—CH_3\end{array}\right. \quad (o\text{- and } p\text{-})\ (IX, X)$$

$$C_6H_4\left<\begin{array}{l}NH—CO—NHPh\\NH—CS—NHPh\end{array}\right. \qquad (XI)$$

$$C_6H_4\left<\begin{array}{l}NH—CO—NHPh\\NH—CS—NH—C_6H_4\cdot CH_3\end{array}\right. \qquad (XII)$$

$$C_6H_4\left<\begin{array}{l}NH—CO—NHPh\\NH—CS—NH—CH_3\end{array}\right. \qquad (XIII)$$

$$C_6H_4\left<\begin{array}{l}NH—CO—NHPh\\NH—CS—NH—C_3H_5\end{array}\right. \qquad (XIV)$$

$$C_6H_4(NH—CO—NH_2)_2 \qquad (XV)$$

$$C_6H_4(NH—CO—NHPh)_2 \qquad (XVI)$$

The unsymmetrically substituted dithiocarbamides VIII, IX and X have now been obtained by the action of one molecule of mustard oil upon compounds of the diamine with one molecule of a mustard oil, thus :

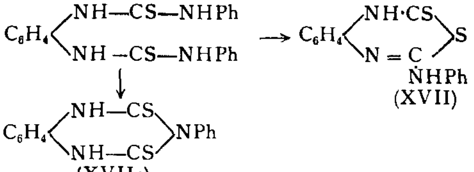
$$C_6H_4\left<\begin{array}{l}NH_2\\NH_2\end{array}\right. \xrightarrow{RNCS} C_6H_4\left<\begin{array}{l}NH—CS—NHR\\NH_2\end{array}\right. \xrightarrow{R'NCS} C_6H_4\left<\begin{array}{l}NH—CS—NHR\\NH—CS—NHR'\end{array}\right.$$

whereas the monothiodicarbamides, XI, XII, XIII and XIV have been prepared by the action of phenyl *iso*cyanate upon compounds of the diamine with one molecule of a mustard oil, thus :

$$C_6H_4\left<\begin{array}{l}NH—CS—NHR\\NH_2\end{array}\right. \xrightarrow{PhNCO} C_6H_4\left<\begin{array}{l}NH—CS—NHR\\NH—CO—NHPh\end{array}\right.$$

o-Phenylenediphenyldithiocarbamide (I) loses a molecule of aniline on being heated with strong hydrochloric acid or 20 per cent. potassium hydroxide solution. The elimination of aniline might take place in either of the following two different ways forming 2-anilino-4 : 5-benzo-7-thioketo-1 : 3 : 6-heptathiodiazine (XVII) or

1-*N*-phenyl-2 : 7-dithioketo-4 : 5-benzo-1 : 3 : 6-heptatriazine (XVII*a*), thus :



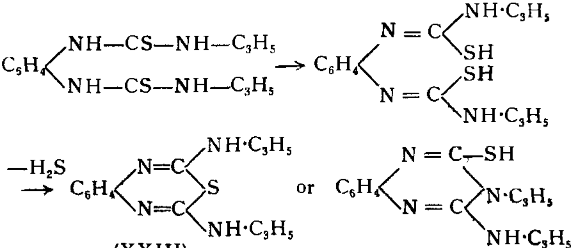
(XVII) (XVII*a*)

The compound actually isolated behaves like a mono-mercaptan as it has been found that for disulphide formation two molecules of the substance require only two atomic proportions of iodine (compare Guha, *J. Amer. Chem. Soc.*, 1922, **44**, 1502). So, the dimercaptanic formula has been rejected. The other disubstituted dithiocarbamides (II-VI and VIII-X) resemble the diphenyl compound in behaviour, yielding substituted aminoheptathiodiazines XVII—XXII, XXIV—XXVI respectively.

R=Ph, XVII ; *o*-tolyl, XVIII ; *p*-tolyl, XIX ; xylyl, XX ; Me XXII ; C_3H_5, XXIV ; XXV=XVIII ; XXVI=XIX.

It is significant that although in compounds VIII, IX, and X the two thiocarbamide groupings are substituted by two different groups (*viz.*, C_6H_5 : C_3H_5, C_6H_5 : *o*-$C_6H_4{\cdot}CH_3$, C_6H_5 : *p*-$C_6H_4{\cdot}CH_3$) aniline is invariably eliminated in each case.

The behaviour of *o*-phenylenediallyldithiocarbamide (VII) is peculiar and instead of allylamine one molecule of sulphuretted hydrogen is eliminated with the formation of 2 : 7-diallylamino-4 : 5-benzo-1 : 3 : 6-thioheptadiazine, XXIII, thus :

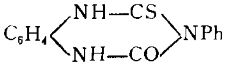


(XXIII)

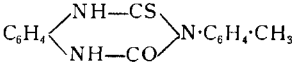
The alternative formula has been rejected on the ground of its showing no mercaptanic properties and also because it forms a diacetyl derivative from which no acetyl group can easily be removed by hydrolysis as would have been the case with an acetylthiol compound (*vide* Guha, *ibid*). Acetic anhydride brings about the same type of ring-closure yielding the diacetyl derivative of XXIII which can be deacetylated only by boiling with strong hydrochloric acid. This peculiar behaviour of the allyl compounds, *viz*., favouring the formation of sulphur-containing rings will also be noticed in the case of mono-allylthiocarbamide, XXXVI.

The action of strong hydrochloric acid on the four phenylene mono-thiodicarbamides XI, XII, XIII and XIV has been studied with the result that they have been found to yield types of compounds quite different from those obtained from the compounds containing dithiocarbamide groupings.

Compounds XI and XII, which are substituted by aryl groups, give 1-*N*-phenyl-2-keto-4 : 5-benzo-7-thioketo-1 : 3 : 6-heptatriazine (XXVII) and 1-*N*-tolyl-2-keto-4 : 5-benzo-7-thioketo-1 : 3 : 6-heptatriazine (XXVIII) respectively.

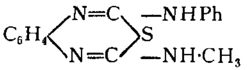


(XXVII)

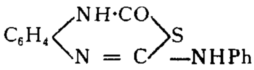


(XXVIII)

The mono-thiodicarbamide (XIII) yields with the loss of one molecule of water 2-methylamino-4 : 5-benzo-7-phenylamino-1 : 3 : 6-thioheptadiazine (XXIX), whereas from (XIV) there is obtained 2-phenylamino-4 : 5-benzo-7-keto-1 : 3 : 6-thioheptadiazine (XXX).

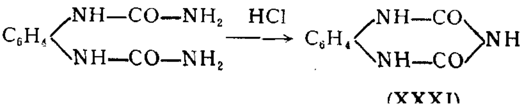


(XXIX)



(XXX)

Phenylenediurea (XV) yields phenylenebiuret (XXXI), whereas the corresponding diphenyl substituted compound (XVI) remains unaffected by the action of hydrochloric acid.



(XXXI)

The first four of the following five mustard oil compounds of *o*-phenylenediamine, *viz.*,

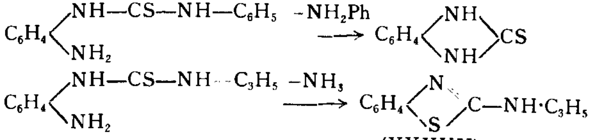
$NH_2{\cdot}C_6H_4{\cdot}NH{\cdot}CS{\cdot}NHPh$ (XXXII),

$NH_2{\cdot}C_6H_4{\cdot}NH{\cdot}CS{\cdot}NH{\cdot}C_6H_4{\cdot}CH_3$ (*ortho* and *para*) (XXXIII and XXXIV),

$NH_2{\cdot}C_6H_4{\cdot}NH{\cdot}CS{\cdot}NH{\cdot}CH_3$ (XXXV),

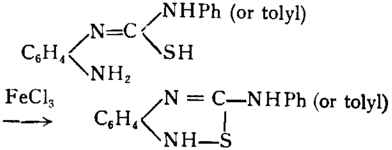
and $NH_2{\cdot}C_6H_4{\cdot}NH{\cdot}CS{\cdot}NH{\cdot}C_3H_5$ (XXXVI)

react with strong hydrochloric acid to yield *o*-phenylenethiourea, evidently with the elimination of the respective amines, whereas, the last one behaves in a quite different manner to yield 5-allylamino-2 : 3-benzo-1 : 4-thiazole (XXXVII), thus :



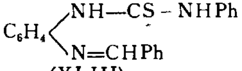
(XXXVII)

A suitable reagent for building up arylaminobenzothiodiazine compounds has been found in ferric chloride, which acting upon (XXXII) and (XXXIV) gives rise to 3 : 4-benzo-6-anilino-1 : 2 : 5-thiodiazine (XXXVIII) and 3 : 4-benzo-6-*p*-tolylamino-1 : 2 : 5-thiodiazine (XXXIX) respectively thus :

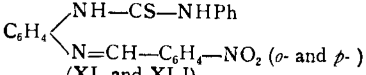


(XXXVIII, XXXIX)

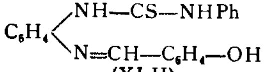
The free amino-groups of the mustard oil compounds of phenylenediamine react readily with aldehydes to yield



(XLIII)

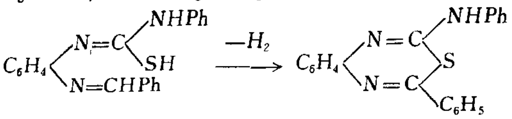


(XL and XLI)



(XLII)

of which the first one has been oxidised by ferric chloride to 2-phenyl-4 : 5-benzo-7-anilino-1 : 3 : 6-heptathiodiazine (*XLIV*), thus :



(**XLIV**)

EXPERIMENTAL.

2-Phenylamino-4 : 5-phenylene-7-thioketo-1 : 3 : 6-thioheptadiazine (XVII).—*o*-Phenylenediphenyldithiocarbamide (2 g., prepared according to the method given in *Annalen*, 1885, **228**, 200) was heated under reflux with hydrochloric acid (65 c.c., *d* 1·19) for about half an hour when a green solution mixed with some tarry matter was obtained. On further heating for 15 minutes, the tarry mass solidified. The reaction mixture was allowed to cool and the green precipitate filtered, washed with water and finally crystallised from water acidulated with a few drops of hydrochloric acid, separating in beautiful white rectangular plates, m.p. 290-291°. It can be precipitated by acid from a caustic soda solution. Yield 1·1 g. (Found : S, 22·61. $C_{14}H_{11}N_3S_2$ requires S, 22·45 per cent.). The presence of aniline in the acid filtrate was proved.

The acetyl derivative.—The above compound (0·5 g.) was heated with about 5 c.c. of acetic anhydride under reflux for about 15 minutes, when a perfectly clear solution was obtained, which, on being poured into cold water, yielded a white crystalline mass. It was further crystallised from acetic acid, m.p. 160°.

The disulphide.—Iodine dissolved in potassium iodide was added drop by drop to an alcoholic solution of the above thio-compound (XVII) and gently warmed. On dilution with water it gave a yellowish amorphous mass which was washed free from excess of iodine with potassium iodide solution, water and finally with alcohol; m.p. 210° (0·067 g. of the substance required 0·0312 g. of iodine for oxidation. $C_{14}H_{11}N_3S_2$ with one thiol group requires 0·03 g. of iodine ; with two thiol groups it requires 0·06 g. of iodine).

Action of caustic potash solution on o-*phenylenediphenyldithiocarbamide : Formation of Compound* (XVII).—*o*-Phenylenediphenyldithiocarbamide (2 g.) was heated under reflux with excess of potassium hydroxide solution (20 per cent.) for about 2 hours when a clear solution was obtained. The solution after cooling was acidified with hydrochloric acid when a white solid came down with slight evolution of sulphuretted hydrogen. The white precipitate after

filtration was crystallised from hot water acidulated with a few drops of hydrochloric acid; m. p. 290-291°. It is soluble in cold dilute alkali and is precipitated by acids. It gives an insoluble mercaptide with mercuric chloride and a disulphide (m.p. 210°) with iodine (Found: S, 22·59. $C_{14}H_{11}N_3S_2$ requires S, 22·45 per cent.). The identity of this compound with (XVII), obtained on treatment with hydrochloric acid, was confirmed by the mixed melting point.

o-*Phenylenedi*-o-*tolyldithiocarbamide* (II).—An alcoholic solution of *o*-phenylenediamine (2·1 g.) was heated under reflux on the water-bath with *o*-tolyl mustard oil (3 g.) for about half an hour when a white crystalline precipitate was obtained and recrystallised from alcohol; m.p. 161°. Yield is almost quantitative (Found: S, 15·62. $C_{22}H_{22}N_4S_2$ requires S, 15·76 per cent.).

2-o-*Tolylamino*-4: 5-*phenylene*-7-*thioketo*-1: 3: 6-*thioheptadiazine* (XVIII).—Two gms. of the above compound (II) were heated under reflux with hydrochloric acid (60 c.c., *d* 1·19) for about 30 minutes when a green solution with tarry matter was obtained. On further heating, the tarry mass solidified and was crystallised from alcohol; m.p. 300° (Found: S, 21·31. $C_{15}H_{13}N_3S_2$ requires S, 21·40 per cent.). The *acetyl* derivative was crystallised from acetic acid; m.p. 200°.

o-*Phenylenedi*-p-*tolyldithiocarbamide* (III).—The method of preparation was the same as in the case of the preceding compound (II). The yield was almost quantitative. It crystallised from alcohol in shining white plates, m.p. 178° (Found: S, 15·82. $C_{22}H_{22}N_4S_2$ requires S, 15·76 per cent.).

2-p-*Tolylamino*-4: 5-*phenylene*-7-*thioketo*-1: 3: 6-*thioheptadiazine* (XIX).—The method of preparation was the same as in the case of the preceding compounds (XVII and XVIII). It was crystallised from alcohol; m.p. 300.° It is soluble in cold dilute alkali from which it is precipitated by acids (Found: S, 21·26. $C_{15}H_{13}N_3S_2$ requires S, 21·40 per cent.). The *disulphide* was prepared as in the case of the corresponding phenyl compound; m.p. 190° (0·206 g. of the substance required 0·088 g. of iodine for oxidation. $C_{15}H_{13}N_3S_2$ containing one thiol group requires 0·087 g. of iodine). The *acetyl* derivative was obtained in the usual way and was crystallised from acetic acid; m.p. 200.°

o-*Phenylenedi*-(1: 3: 4)-*xylyldithiourea* (IV).—The method of preparation was the same as in the case of the corresponding phenyl and tolyl compounds. It was crystallised from alcohol; m.p. 145° (Found: S, 14·39. $C_{24}H_{26}N_4S_2$ requires S, 14·74 per cent.).

Action of strong hydrochloric acid upon (IV): *Formation of* 2-(1: 3: 4)-*xylylamino*-4: 5-*phenylene*-7-*thioketo*-1: 3: 6-*thioheptadiazine*

(XX).—This was prepared similarly to the compounds (XVII, XVIII, XIX) and was crystallised from alcohol; m.p. 295°. It is soluble in cold dilute alkali from which it can be precipitated by acids. It gave an amorphous *disulphide* (m.p. 172°) with iodine solution (Found: S, 20·13. $C_{16}H_{15}N_3S_2$ requires S, 20·44 per cent.). The *acetyl* derivative was crystallised from acetic acid; m.p. 296°.

o-*Toluylenediphenyldithiocarbamide* (V).—An alcoholic solution of *o*-toluylenediamine (1·2 g.) was heated under reflux on the water-bath with phenyl mustard oil (2·7 g.) for about 30 minutes when a white crystalline precipitate was obtained which was crystallised from alcohol; m.p. 142°. Yield is almost quantitative (Found: S, 16·48. $C_{21}H_{20}N_4S_2$ requires S, 16·32 per cent.).

Action of strong hydrochloric acid upon (V): *Formation of* 2-*Phenylamino*-4: 5-*methylbenzo*-7-*thioketo*-1 : 3: 6-*thioheptadiazine* (XXI).—The above compound (V, 2 g.) was heated under reflux with concentrated hydrochloric acid (60 c.c.) for about half an hour when a green solution was obtained, and on cooling deposited a solid which was crystallised from alcohol, m.p. 265°. It is soluble in cold dilute alkali from which it is precipitated by acids (Found: N, 14·21. $C_{15}H_{13}N_3S_2$ requires N, 14·04 per cent.). The presence of aniline in the acid filtrate was proved. The *disulphide* was prepared in the usual way; m.p. 137°. The *acetyl* derivative was crystallised from acetic acid, m.p. 185°.

o-*Phenylenedimethyldithiocarbamide* (VI).—Phenylenediamine (3 g.) was dissolved in alcohol to which methyl mustard oil (4 g.) was added and the solution boiled for some time. The clear solution, on cooling, gave colourless crystals which were recrystallised from alcohol, m. p. 175° (Found: S, 24·85. $C_{10}H_{14}N_4S_2$ requires S, 25·19 per cent.).

2-*Methylamino*-4 : 5-*benzo*-7-*thioketo*-1 : 3 : 6-*thioheptadiazine* (XXII)—Two grams of the compound (VI) were boiled with strong hydrochloric acid (65 c. c.) for about 2 hours. The solution, when cold, gave a white crystalline mass which was further crystallised from alcohol; m. p. 168°. It is soluble in cold alkali from which it is precipitated by acids (Found: N, 19·15. $C_9H_9N_3S_2$ requires N, 18·83 per cent.). The *disulphide* was prepared in the usual way; m.p. 194°.

2 : 7-*Diallylamino*-4 : 5-*benzo*-1 : 3 : 6-*thioheptadiazine* (XXIII).—Three g. of *o*-phenylenediallylthiourea (VII) (prepared according to the method given in *Annalen*, 1885, **228**, 201) were heated with strong hydrochloric acid (70 c. c.) under reflux for about 30 minutes when a greenish coloured clear solution was obtained. The cold solution, on dilution with water, gave no precipitate, but on making alkaline with ammonia a white solid mixed with tarry matter was obtained, and was

purified by dissolving in diltue hydrochloric acid and precipitating with ammonia. It was finally crystallised from acetone, m. p. above 300° (Found: N, 20·37. $C_{14}H_{16}N_4S$ requires N, 20·58 per cent.)

Action of acetic anhydride upon o-*Phenylenediallyldithiocarbamide: Formation of diacetyl compound of* (XXIII).—*o*-Phenylenediallyldithiourea (2 g.) was heated with 10 c. c. of acetic anhydride under reflux for about 45 minutes when a clear solution was obtained and on cooling gave a crystalline mass. It was further crystallised from acetic acid in slender white needles, m. p. 185° (Found: N, 16·11. $C_{18}H_{20}N_4S$ requires N, 15·73 per cent.). Deacetylation was effected by boiling with strong hydrochloric acid for about half an hour when a clear solution was obtained, and on being made alkaline gave a white precipitate which crystallised from acetone, m. p. above 300° (Found: N, 20·39 $C_{14}H_{16}N_4S$ requires N, 20·58 per cent.).

o-*Phenylene*-sym-*phenylallyldithiourea* (VIII).—An alcoholic solution of 1-*o*-aminophenyl-3-phenylthiocarbamide (2·5 g.) and allyl mustard oil (1 g.) was heated on the water-bath under reflux for about 2 hours. The reaction product was allowed to cool when a crystalline mass separated and was further crystallised from alcohol, m. p. 245° (Found: S, 18·48. $C_{17}H_{18}N_4S_2$ requires S, 18·71 per cent.)

2-*Allylamino*-4:5 *benzo*-7-*thioketo*-1 : 3 : 6-*thioheptadiazine*(XXIV).—Two g. of the compound (VIII) were heated with strong hydrochloric acid (50 c. c.) under reflux for about an hour. The solution became red with the separation of a solid which crystallised from alcohol, m.p. 293°. It is soluble in cold dilute alkali from which it can be precipitated by acids. The presence of aniline in the acid filtrate was proved (Found: N, 16·58. $C_{11}H_{11}N_3S_2$ requires N, 16·86 per cent.). The *disulphide* was prepared as usual; m. p. above 300°.

o-*Phenylene*-sym-*phenyl*-o-*tolyldithiourea* (IX).—An alcoholic solution of 1-*o*-aminophenyl-3-phenylthiocarbamide (2·4 g.) and *o*-tolyl mustard oil (1·5 g.) was heated on the water-bath under reflux for about 2 hours. The product on cooling gave a solid which crystallised from alcohol; m. p. 136° (Found: S, 16·59. $C_{21}H_{20}N_4S_2$ requires S, 16·32 per cent.).

2-o-*Tolylamino*-4 : 5-*phenylene*-7-*thioketo*-1 : 3 : 6-*thioheptadiazine* (XXV).—Two g. of the compound (IX) were heated with strong hydrochloric acid (50 c. c.) under reflux for about an hour when a clear solution was obtained. This, on being further heated, gave a solid mass which was crystallised from alcohol; m. p. 300°. It is soluble in cold alkali from which it is precipitated by acids. The presence of aniline in the acid filtrate was proved (Found: N, 14·31. $C_{15}H_{13}N_3S_2$

requires N, 14·04 per cent.). *Identity of this compound with the compound (XVIII) was further confirmed by the mixed melting point.*

o-*Phenylene*-sym-*phenyl.*-p-*tolyldithiourea* (X).—The method of preparation was the same as in the case of the compound (IX). The substance was crystallised from alcohol; m. p. 165° (Found: S, 16·48. $C_{21}H_{20}N_4S_2$ requires S, 16·32 per cent.).

2-p-*Tolyamino*-4: 5-*phenylene*-7-*thioketo*-1: 3: 6-*thioheptadiazine* (XXVI).—The method of preparation was the same as in the case of the compound (XXV). The substance was crystallised from alcohol; m. p. 300° (Found: N, 14·28. $C_{15}H_{13}N_3S_2$ requires N, 14·04 per cent.). The identity of this compound with (XIX) was further confirmed by taking a mixed melting point.

1-*Phenylcarbamido*-2-*phenylthiocarbamidobenzene* (XI.)—An alcoholic solution of 1-*o*-aminophenyl-3-phenylthiocarbamide (2·4 g.) and phenyl *iso*cyanate (1·2 g.) was heated for about 30 minutes when a white solid separated and was crystallised from alcohol; m. p. 200° (after shrinking at 140°). (Found: N, 15·27. $C_{20}H_{18}ON_4S$ requires N, 15·46 per cent.).

1-*Phenyl*-2-*keto*-4: 5-*benzo*-7-*thioketo*-1: 3-6-*heptatriazine* (XXVII).—Two grams of the compound (XI) were heated under reflux with concentrated hydrochloric acid (60 c.c.) for about 2 hours when a solid mass with a greenish coloured mother-liquor was obtained. The solid was filtered, washed with water and crystallised from hot alcohol: m.p. 185°. It is soluble in cold ditute alkali from which it is precipitated by acids (Found: N, 15·30. $C_{14}H_{11}ON_3S$ requires N, 15·61 per cent.). The presence of aniline in the mother-liquor was proved.

The *disulphide* was obtained in the usual manner; m.p. 128-130°.

1-*Phenylcarbamido*-2-p-*tolyl-thiocarbamidobenzene* (XII).—An alcoholic solution of 1-*o*-aminophenyl-3-*p*-tolylthiocarbamide (5 g.) and phenyl *iso*cyanate (2·7g.)was heated for about 30 minutes when a white solid separated and was crystallised from alcohol; m. p. 165° (Found: N, 14·69. $C_{21}H_{20}ON_4S$ requires N, 14·97 per cent.).

1-p-*Tolyl*-2-*keto*-4: 5-*benzo*-7-*thioketo*-1: 3: 6-*heptatriazine* (XXVIII).—The method of preparation was the same as in the case of the compound (XXVII). The product was crystallised from hot alcohol; m. p. 175-176°. It is soluble in cold alkali from which it is precipitated by acids (Found: N, 14·67. $C_{15}H_{13}ON_3S$ requires N, 14·79 per cent).

The *disulphide* was obtained in the usual manner; m. p. 164°.

1-*Phenylcarbamido-2-methylthiocarbamidobenzene* (XIII).—An alcoholic solution of 1-*o*-aminophenyl-3-methylthiocarbamide (3 g.) and phenyl *iso*cyanate (2·4 g.) was heated for about half an hour when a white solid separated and was crystallised from alcohol; m. p. 98° (Found: N, 18·38. $C_{15}H_{16}ON_4S$ requires N, 18·67 per cent).

1-*Phenylamino*-3 : 4-*benzo*-6-*methylamino*-2 : 5 : 7-*thioheptadiazine* (XXIX).—Two grams of the compound (XIII) were heated under reflux with strong hydrochloric acid (60 c.c.) for about 2 hours. The clear solution, on cooling, gave a colourless crystalline mass which was recrystallised from alcohol; m. p. 195°. It is soluble in hydrochloric acid but insoluble in alkali (Found: N, 18·78. $C_{15}H_{14}N_4S$ requires N, 19·14 per cent.).

1-*Phenylcarbamido-2-allylthiocarbamidobenzene* (XIV).—The method of preparation was the same as in the case of the compound (XIII). The product was crystallised from alcohol; m. p. 160° (Found: N, 17·41. $C_{17}H_{18}ON_4S$ requires N, 17·17. per cent.).

1-*Phenylamino*-3 : 4-*benzo*-6-*keto*-2 : 5 : 7-*thioheptadiazine* (XXX). —Two grams of the compound (XIV) were heated with strong hydrochloric acid under reflux for about 2 hours. The clear solution on cooling, gave a crystalline mass which was recrystallised from hot water acidulated with a few drops of hydrochloric acid. It is soluble in hydrochloric acid but insoluble in alkali (Found: N, 12·46; S, 9·05. $C_{14}H_{11}ON_3S$, $3H_2O$ requires N, 12·39; S, 9·4 per cent.).

2 :7-*Diketo*-4: 5-*benzo*-1: 3: 6-*heptatriazine* (XXXI).—*o*-Phenylenediurea (XV) prepared according to the method given in *Ber.*, 1883, **16** 592; 2 g.) was heated under reflux with strong hydrochloric acid for about 40 minutes when a greenish black solution was obtained. The solution, on cooling, deposited crystals which were recrystallised from hot water acidulated with a few drops of hydrochloric acid. It sublimed slowly without fusion above 300° into shining mica-like plates, and is soluble in cold alkali from which it is precipitated by acids (Found: N, 19·90. $C_8H_7N_3O_2$, $2H_2O$ requires N, 19·71 per cent.)

The *acetyl* derivative was crystallised from acetic acid; m.p. 190°.

o-*Phenylene*-sym-*diphenylcarbamide* (XVI).—Phenyl *iso*cyanate (2·3 g.) was added to an alcoholic solution of *o*-phenylenediamine (1 g.) and the solution boiled for sometime when a colourless crystalline mass came out which crystallised from alcohol: m. p. 220° (Found: N, 15·99. $C_{20}H_{18}O_2N_4$ requires N, 16·18 per cent.). Action of strong hydrochloric acid produced no change.

Action of strong hydrochloric acid upon (XXXII).—*Formation of o-Phenylenethiourea.*—1-*o*-Aminophenyl-3-phenylthiocarbamide, prepared

according to the method of Lellmann (*Annalen*, 1885, **228**, 212) was heated with strong hydrochloric acid under reflux for about 10 minutes when a clear solution was obtained. The solution was further boiled for about 40 minutes when greenish white crystals came out, which crystallised from hot water acidulated with a few drops of hydrochloric acid; m.p. 301–302°. Lellmann (*Annalen*, 1884, **221**, 14) gave 290° as the m.p. of this compound (Found: N, 18·42. $C_7H_6N_2S$ requires N, 18·66 per cent.). The presence of aniline in the acid filtrate was proved.

The *disulphide* prepared in the usual manner melted at 230°.

The *acetyl* derivative melted at 200°.

1-o-*Aminophenyl*-3-o-*tolylthiocarbamide* (XXXIII).—It was prepared in the same manner as the phenyl compound. It was crystallised from benzene; m.p. 160° (Found: N, 16·18. $C_{14}H_{15}N_3S$ requires N, 16·34 per cent.). Like the corresponding phenyl compound (XXXII), it gave *o*-phenylenethiourea by the action of strong hydrochloric acid.

1-*o*-*Aminophenyl*-3-p-*tolylthiocarbamide* (XXXIV).—It was also prepared in the same manner as the corresponding phenyl compound (XXXII) and crystallised from benzene in shining white crystals, m.p. 146–147° (Found: N, 16·14. $C_{14}H_{15}N_3S$ requires N, 16·34 per cent.). It also gave *o*-phenylenethiourea with strong hydrochloric acid.

1-o-*Aminophenyl*-3-*methylthiocarbamide* (XXXV).—It was prepared in the same manner as the phenyl and tolyl compounds and was crystallised from benzene; m.p. 117° (Found: N, 23·07. $C_8H_{11}N_3S$ requires N, 23·20 per cent.). It also gave *o*-phenylenethiourea (m.p. 301–302°) with strong hydrochloric acid.

1-o-*Aminophenyl*-3-*allylthiocarbamide* (XXXVI).—It was also prepared similarly to the phenyl and tolyl compounds and was crystallised from benzene; m.p. 115° (Found: N, 20·04. $C_{10}H_{13}N_3S$ requires N, 20·28 per cent.).

Action of strong hydrochloric acid upon (XXXVI): *Formation of* 5-*Allylamino*-2 : 3-*benzo*-1 : 4-*thiazole* (XXXVII).—The compound (XXXVI; 1·5 g.) was heated under reflux with strong hydrochloric acid (55 c.c.) for about an hour giving a clear solution which, on cooling, and dilution with water, gave no precipitate; it was neutralised with dilute caustic soda under cooling when a white shining crystalline mass came out which was further crystallised from alcohol; m.p. 180° (Found: S, 16·52. $C_{10}H_{10}N_2S$ requires S, 16·84 per cent.).

The *acetyl* derivative melted at 198°.

Action of ferric chloride upon (XXXII) : *Formation of* 6-*Phenylamino*-3 : 4-*benzo*-1 : 2 : 5-*thiodiazine* (XXXVIII).—Excess of ferric chloride solution was added to 1-*o*-aminophenyl-3-phenylthiocarbamide (3 g.) and the mixture heated under reflux for about an hour; when cooled, a yellow solid mass mixed with some black tarry matter separated. After the removal of the tarry matter with steam the residual solid crystallised from alcohol in yellow slender needles; m.p. 155–156°. It is insoluble in alkali but soluble in hot hydrochloric acid (Found : S, 13·13. $C_{13}H_{11}N_3S$ requires S, 13·27 per cent.).

6-p-*Tolylamino*-3 : 4-*benzo*-1 : 2 : 5-*thiodiazine* (XXXIX).—The method of preparation was the same as in the case of the previous compound (XXXVIII). It was crystallised from alcohol; m.p. 93° (Found : S, 12·13. $C_{14}H_{13}N_3S$ requires S, 12·54 per cent.).

1-o-*Nitrobenzalanilino*-3-*phenylthiocarbamide* (XL).—An alcoholic solution of 1-*o*-aminophenyl-3-phenylthiocarbamide (2·5 g.) and *o*-nitrobenzaldehyde (1·5 g.) was heated on a water-bath under reflux for half an hour. On cooling, orange-coloured crystals gradually separated and were recrystallised from alcohol, m.p. 215° (Found : N, 15·17. $C_{20}H_{16}O_2N_4S$ requires N, 14·89 per cent.).

1-m-*Nitrobenzalanilino*-3-*phenylthiocarbamide* (XLI).—To a hot alcoholic solution of 1-*o*-aminophenyl-3-phenylthiocarbamide (2·5 g.) an alcoholic solution of *m*-nitrobenzaldehyde (1·5 g.) was added when at once a yellowish white crystalline mass came out which crystallised from alcohol, m.p. 153-154° (Found : N, 15·21. $C_{20}H_{16}O_2N_4S$ requires N, 14·89 per cent.).

1-o-*Hydroxybenzalanilino*-3-*phenylthiocarbamide* (XLII).—It was prepared similarly to the foregoing compound and was crystallised from alcohol; m.p. 180° (Found : N, 12·05. $C_{20}H_{17}ON_3S$ requires N, 12·10 per cent.).

1-*Benzalamino*-3-*phenylthiocarbamide* (XLIII.)—It was prepared similarly to the compound (XLI); m.p. 265-267° (Found : N, 12·92. $C_{20}H_{17}N_3S$ requires N, 12·68 per cent.).

Action of ferric chloride upon (XLIII) : *Formation of* 2-*Phenyl*-4 : 5-*benzo*-7-*phenylamino*-1 : 3 : 6-*thioheptadiazine* (XLIV).—1-Benzalanilino-3-phenylthiocarbamide (1·5 g.) was heated under reflux with an excess of ferric chloride solution for an hour when a yellow solid mixed with some tarry matter was obtained. After removing the tarry matter with steam, the solid was crystallised from alcohol; m.p. 105°. It is insoluble in alkali but soluble in hot acids (Found : N, 12·58. $C_{20}H_{15}N_3S$ requires N, 12·76 per cent.).

Phenylthiocarbamidophenylurethane, $CO_2Et{\cdot}NH{\cdot}C_6H_4{\cdot}NH{\cdot}CS{\cdot}NHPh$.—To a hot benzene solution of 1-*o*-aminophenyl-3-phenylthiocarbamide (2·4 g.) chlorcarbonic ester (1·1 g.) was added. The reaction took place at once and the separated white solid crystallised from alcohol; m.p. 288-290° (Found: N, 13·13. $C_{16}H_{17}O_2N_3S$ requires N, 13·33 per cent.). Strong hydrochloric acid was without action.

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OIL FROM THE SEEDS OF *SAPINDUS TRIFOLIATUS* (LINN.).

By D. R. Paranjpe and P. Ramaswami Ayyar.

Sapindus trifoliatus (N. O. *Sapindaceæ*), the soapnut tree, is found in the villages of Southern India and Ceylon, and cultivated in Bengal for the fruit. It has long been used by the Hindus as a detergent, and is preferred to soap for certain purposes (*Pharm. Indica*, vol. 1, 368). The vernacular names of the tree are: Hindi and Mahratti, *Ritha*; Guzrathi, *Aritha*; Telugu, *Kunkudukayalu*; Kanarese, *Artala*; Tamil, *Poovandi.* While the fruit is valued principally for the high saponine content of its pericarp, it appeared of interest to examine also the oil contained in the seeds.

The fruits are fleshy, two or three united, and each is the size of a cherry. The seeds resemble the fruit in shape, are black, sub-spheroid, smooth, and have the size of a large pea. The fruits used in the present investigation were obtained locally and were separated into pericarp (65·7 per cent.) and seeds (34·3 per cent.). The latter were further divided into one part of yellowish green kernels and two parts of outer shell. The finely powdered seed-kernels on extraction with petroleum ether gave 44·7 per cent. of a dark yellow oil which deposits a small quantity of stearin on standing.

An examination of the oil in the usual manner has revealed the remarkable fact that in addition to the glycerides of palmitic, stearic, oleic and lignoceric acids, the oil contains a high proportion (22 per cent.) of *n*-eicosanic acid. The oil may be classed as a non-drying oil from the low iodine value (58·5).

EXPERIMENTAL.

The analytical constants of the oil given in Table I were determined in the usual way and compare well with those of A. K. Menon (*J. Soc. Chem. Ind.*, 1910, 1431), whose results appear in the second column.

TABLE I.

Sp. gr. $\frac{100°}{100°}$	0·8540	0·8542
$n_D 25°$	1·4764	1·4748
Saponification value	194·1	191·8
Iodine value	58·5	58·6
Reichert-Meisel value	1·5	1·6
Acetyl value	0·0	0·0
Unsaponifiable matter	1·2 per cent.	1·1 per cent.
Hehner value	93·5	93·9

The Mixed Fatty Acids.

For the preparation of the fatty acids, the oil was saponified with an alcoholic solution of sodium hydroxide, the dried soap extracted with ether to remove unsaponifiable matter, and the fatty acids regenerated from the soap by boiling with 1 : 1 diluted hydrochloric acid. The acids obtained after taking up in ether and removing the solvent had the constants given in Table II, the second column showing those of A. K. Menon.

TABLE II.

Saponification value	195·4	188·6
Iodine value	61·3	57·0
Equivalent weight	287·0	297·3
Titre	47°	54·4° (m. p.)

The mixed acids were separated into the saturated and unsaturated acids by the method of Twitchell (*J. Ind. Eng. Chem.*, 1921, **13**, 806), the process being repeated twice. The separated acids were analysed with results summarised in Table III.

TABLE III.

—	Saturated acids (38·5 per cent.)	Unsaturated acids (61·5 per cent.)
Iodine value	1·1	86·2
Equivalent weight	292·5	283·0
n_D 60°	1·4400	1·4450

Identification of the Unsaturated Acids.

The additive bromo-compounds were prepared by the method of Jamieson and Baughman (*J. Amer. Chem. Soc.*, 1920, **42**, 2398). No hexabromides separated at zero nor was any crystalline tetrabromide formed from petroleum solution. The solvent was therefore distilled and bromine estimated in the residual oily bromide (Br, 36·2. Calc. for dibromide of oleic acid, Br, 36·2 per cent.). The unsaturated acid was therefore assumed to be oleic acid.

Identification of the Saturated Acids.

The saturated acids recovered from the insoluble lead salts were converted into the methyl esters by boiling with ten times their volume of anhydrous methyl alcohol containing 4 per cent. of hydrogen chloride. The purified esters were twice fractionated under a pressure of 6 mm. and twelve fractions were obtained. The main results of the fractionation are summarised in Table IV, and the detailed examination of each fraction is given below.

TABLE IV.

Fraction No.	B. p. 6 mm.	Yield in grams	Titre of ester	M. W. of ester	M. p. of acid
I	below 190°	9·7	25·4°	285·5	54·5°
II	191–196	11·7	28·4	289·1	53–54
III	197–198	12·0	31·2	297·0	55–56
IV	199–206	14·5	33·6	305·5	57–58
V	206–207	20·1	36·8	315·2	67
VI	207–208	17·1	38·5	320·2	70
VII	208–209	10·4	39·8	322·3	...
VIII	209–210	10·0	41·2	325·3	73
IX	211–217	11·8	42·0	329·0	74
X	218–225	13·4	43·0	336·7	71
XI	226–235	5·2	44·6	335·2	69–70
XII	...	1·1	...	...	80·5

Fraction I was a liquid. The acids obtained from it were crystallised from 70 per cent. alcohol, when an acid melting at 73° separated; and from the mother liquor, a solid acid melting at 53–54° was obtained having an equivalent weight of 268. Assuming that the acid melting at 73° was *n*-eicosanic acid which was identified in the later fractions and that the yield was approximately quantitative, it was possible to calculate from the molecular weight that the fraction was a ternary mixture of palmitic ester (5·1 g.), stearic ester (3·8 g.) and *n*-eicosanic ester (0·8 g.).

Fraction II.—The ester (6·2 g.) was saponified with alcoholic potash, neutralised with dilute hydrochloric acid so as to make about an 80 per cent. alcohol and allowed to stand overnight. This

precipitated the acid of high molecular weight as potassium soap, from which the acid was regenerated and crystallised from acetone; 0·76 g. was obtained, and having m.p. 73–74° with equivalent, 310 was proved to be different from Kahlbaum's arachidic acid (mixed melting point, 63–65°), but identical with a synthetic specimen of n-eicosanic acid (mixed m. p., 73–75°). Acid (m. p. 50° with equivalent, 270) from the soluble potassium soap was resolved by fractional precipitation with lead acetate in 90 per cent. alcoholic solution, into (*a*) an acid (equivalent, 282; m. p. 69–70°, unchanged on mixing with stearic acid), (*b*) an acid m. p. 53–54° and (*c*) an acid m. p. 59–60° (unchanged on mixing with palmitic acid) having an equivalent weight of 251 (palmitic acid, 256). Assuming that the acid melting at 73–74°, is a mixture of *n*-eicosanic and stearic acids, and that all the former acid was precipitated, the composition of this fraction is palmitic ester (5·1 g.), stearic ester (5·1 g.) and *n*-eicosanic ester (1·5 g.).

Fraction III.—The ester (7·1 g.) was saponified with potash, and resolved into five fractions by fractional precipitation with an alcoholic solution of magnesium acetate (10 grams in 100 c.c. of 95 per cent. alcohol). The following acids were thus obtained:—

(*a*) 2·4 g., m.p. 72–73° (equivalent, 308·5) after crystallisation from acetone melted at 73–75° (unchanged on mixing with *n*-eicosanic acid, but lowered by 6° on mixing with Kahlbaum's arachidic acid).

(*b*) 0·2 g., m.p. 66–69° (with stearic acid, at 65–66°).

(*c*) 0·5 g., m.p. 65–68° (with stearic acid at 65–66°).

(*d*) 0·7 g., m.p. 60–62° (with palmitic acid at 55–57° and with stearic acid at 60–67°).

(*e*) 2·5 g., m.p. 54–55° with an equivalent of 270; on crystallising this from alcohol, ether and acetone consecutively, two crops of crystals were obtained melting at 58–59° and 59–61°, respectively. By taking mixed melting points of these with specimens of pure palmitic, stearic and synthetic heptadecoic (margaric) acids they were respectively found to be a palmitic-stearic-mixture, and pure palmitic acid. Fraction III therefore consists of palmitic ester (3·3 g.), stearic ester (4·9 g.) and *n*-eicosanic ester (3·8 g.).

Fraction IV.—This was further separated into four portions by refractionation under reduced pressure as shown in Table V. From the molecular weights and melting points of the acids obtained from three of the refractionated portions the mixture should consist of palmitic ester (4·9 g.), stearic (8·4 g.) and *n*-eicosanic ester (1·2 g.).

TABLE V.

Fraction No.	Yield in grams	Titre of ester	M. W. of ester	M.p. of acid
1	2·9	28·2°	278	56°
2	4·0	31·5°	...	...
3	3·9	36·5°	298	67°
4	1·1	40·4°	...	73°

Fraction V.—By careful refractionation and hydrolysis this gave 1·5 g. of pure palmitic acid, nearly 1 g. of stearic acid, and a residual mixture of stearic and *n*-eicosanic acids from which the latter was obtained pure (m.p. 73–75°) by crystallisation from acetone. Its composition is therefore palmitic ester (1·6 g.), stearic ester (2·7 g.) and *n*-eicosanic ester (15·8 g.).

Fractions VI–VIII constitute a mixture of stearic ester (5·2 g.), and *n*-eicosanic ester (32·3 g.).

Fraction IX is principally *n*-eicosanic ester, contaminated with a small proportion of lignoceric ester.

Fractions X and XI are undoubtedly mixtures of *n*-eicosanic and lignoceric esters, because from the residual fraction XII, an acid was prepared which after crystallisation from 98 per cent. alcohol melted at 80–80·5° (unchanged on mixing with pure lignoceric acid, but lowered by 3° on mixing with behenic acid).

Detailed composition of the saturated esters (twelve fractions), summarised in Table VI (grams), shows totals of palmitic ester 14·6 per cent., stearic 21·9 per cent., *n*-eicosanic 56·8 per cent. and lignoceric ester 6·6 per cent.

TABLE VI.

Fraction No.	Palmitic	Stearic	*n*-Eicosanic	Lignoceric
I	5·1	3·8	0·8	...
II	5·1	5·1	1·5	...
III	3·3	4·9	3·8	...
IV	4·9	8·4	1·2	...
V	1·6	2·7	15·8	...
VI	...	3·5	13·6	...
VII	...	1·4	9·0	...
VIII	...	0·3	9·7	...
IX	...	...	10·5	1·3
X	...	...	8·4	5·0
XI	...	...	3·5	1·7
XII	...	...	...	1·1
Total in grams ...	20·0	30·1	77·8	9·1

PREPARATION OF PURE *n*-EICOSANIC AND *n*-HEPTADECOIC ACIDS.

A specimen of the first named acid was prepared from stearic acid by the following series of reactions, adopting the methods given by Adam and Dyer (J.C.S., 1925, **127**, 70) and Levene and Taylor (*J. Biol. Chem.*, 1924, **59**, 916):—

$$C_{17}H_{35}\cdot CO_2H \rightarrow C_{17}H_{35}\cdot CO_2Et \rightarrow C_{18}H_{37}\cdot OH \rightarrow C_{18}H_{37}I$$
$$\rightarrow C_{18}H_{37}\cdot CH(CO_2Et)_2 \rightarrow C_{18}H_{37}\cdot CH(CO_2H)_2 \rightarrow$$
$$C_{18}H_{37}\cdot CH_2\cdot CO_2H \text{ (m.p. } 75^\circ).$$

n-Heptadecoic acid was prepared also from stearic acid by the usual method of degrading higher fatty acids as outlined below (Levene and West, *J. Biol. Chem.*, 1913, **16**, 477) :—

$$C_{17}H_{35}\cdot CO_2H \rightarrow C_{16}H_{33}\cdot CHBr\cdot CO_2H$$
$$\rightarrow C_{16}H_{33}\cdot CH(OH)\cdot CO_2H \rightarrow C_{16}H_{33}\cdot CO_2H \text{ (m.p. } 59\text{–}60^\circ).$$

The unsaponifiable matter yields about 30 per cent. of a sterol melting at 115–120°, which is most probably a mixture, as its alcoholic solution deposits two distinct types of crystals (short prismatic needles, and hexagonal plates) as seen under the microscope.

SUMMARY.

The seed-kernels of *Sapindus trifoliatus* fruits contain 44·7 per cent. of a non-drying fatty oil, the analytical constants of which have been determined.

The oil is composed of the glycerides of palmitic (5·6 per cent.), stearic (8·5 per cent.), *n*-eicosanic (21·9 per cent.), lignoceric (2·5 per cent.) and oleic (61·5 per cent.) acids.

The oil is a rich source of *n*-eicosanic acid.

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I.—AMYLASE FROM *ZEA MAIS*.

By Vinayak Narayan Patwardhan.

This investigation is the second of a series of studies on amylases of vegetable origin, the first one being the amylase from *Sorghum vulgare*. The results obtained from the latter have already been published (Patwardhan and Norris, *J. Indian Inst. Sci.*, 1928, **11A**, 121). The results obtained with amylase from *Zea mais* as well as *Sorghum vulgare* fully warrant the view that it is hazardous to form any definite opinion on the distinct individuality of amylase till amylases from various origins are thoroughly examined.

The proportion of the liquefying and saccharifying components in an enzyme is not constant in the amylases so far examined. The ratio of saccharification to liquefaction in maize amylase exceeds that in barley amylase but is less than that in cholam amylase. The idea of influencing either the saccharification or liquefaction unequally by changing the conditions of reaction has again been followed in the present case. The effect of such changes, however, appears to be practically the same with this enzyme as in the previous cases investigated. The enzyme exerts its optimum saccharifying activity between 55° and 60°. The same can be said about the liquefaction. One fact which is noteworthy, however, is the very slow fall of activity as the temperature rises to 70°. In the case of barley and cholam amylases the activity at 70° is considerably smaller than that at the optimum temperature; but in this case the fall is relatively small. In other words the maize enzyme seems to be less liable to destruction by heat than that derived from cholam.

A study of the effect of change in the hydrogen-ion concentration shows that the enzyme behaves perfectly normally. The saccharifying activity increases slowly and constantly till the P_H 5·0 is reached, a gradual fall following. The region of the maximum liquefying activity is pushed further to the acid side, this being possibly due to the liquefying action of the acid itself which might to a certain extent compensate for the decreasing activity of the enzyme.

The enzyme has been found to pass through parchment membranes, but not through collodion membranes; it shows quite abnormal behaviour after dialysis, the enzyme in collodion bags having lost activity. The period of complete disappearance of activity is variable. In the case of maize-malt extract the activity is almost completely lost in three days, while the precipitated enzyme took longer. The inacti-

vation is such that the activity cannot be restored by any means tried, addition of salts, asparagine and buffer solution having been found unsuccessful. The idea that the enzyme protein might be broken down by proteolytic enzyme possibly present in the maize amylase was also investigated, but the precipitated enzyme was found to be without proteolytic action. The possibility that the dialysate might remove an amylase-component was also tested by adding the concentrated dialysate to the inactivated enzyme and testing the activity, but there was no activation. At present this inactivation remains unexplained, undialysed samples of the enzyme kept under similar conditions remaining active over the same period of time.

EXPERIMENTAL.

Malting and preparation of enzyme.—The grain was steeped in a slow stream of water for 60 hours at room temperature, spread on sand and allowed to germinate in the shade. After the plumules had grown to approximately half-an-inch the seeds were collected and dried in the sun for 6 hours. The sun-dried seeds were further dried in an oven, first at 50° for 24 hours, then at 70° for 24 hours and finally at 100° for 24 hours. After cooling, the malt was separated from dried plumules and radicles, and powdered. The powder could be stored without deterioration.

Dry malt powder (200 gms.) was extracted in cold water with 600 c.c. of 20 per cent. alcohol for 24 hours with frequent shaking. The extract was filtered and the enzyme precipitated from the filtrate by the slow addition of 1200–1300 c.c. of 95 per cent. alcohol with constant stirring. After 12 hours the supernatant liquid was siphoned from the precipitate which was washed successively with 98 per cent. and 99·5 per cent. alcohol and finally with ether, being then dried in vacuum over calcium chloride. The dry precipitate can be conveniently preserved for a long time without losing activity.

Experiment to follow the liquefaction and saccharification of the enzyme.—The liquid contained 50·0 c.c. starch paste (2 per cent.),

No.	Time in mins.	Maltose, mgms.	Viscosity in terms of t (secs.)	Colour with iodine
1	0	0	171·2	Deep blue
2	15	10·0	117·0	Violet
3	30	16·5	111·0	Very faint violet
4	60	24.0	107·2	Brown
5	100	29·0	106·0	Faint brown
6	260	42·5	...	...
7	1440	69·3	...	No change
8	4560	84·1	...	

12·5 c.c. enzyme (0·5 per cent.) and 37·5 c.c. water, 1 c.c. toluene being added for every 100 c.c.

Figures for comparison with the experiments on barley and cholam enzymes are given below :—

Time in hrs.	Saccharification Maltose (mgms.)		Time in hrs.	Liquefaction t in secs.	
	Barley	Cholam.		Barley	Cholam
0	0	0	0	165·5	177·7
15	39·4	3·3	15	109·2	136·0
30	58·0	5·5	30	107·5	128·8
60	69·5	11·4	60	106·2	117·8
100	76·8	18·4	100	106·0	112·0

Experiments to ascertain the temperature effect.—Eight flasks each contained 15 c.c. starch paste (2 per cent.) and 5 c.c. water. At the selected temperature the enzyme (10 c.c., 0·3 per cent. solution) was added and the flask maintained at that temperature. After half-an-hour 10 c.c. portions were removed for determination of sugar and 10 c.c. for precipitation by alcohol. A typical experiment is recorded below.

Temperature, degrees	Maltose, mgms.	Dry precipitate, mgms.
30	17	62·8
40	21·4	70·8
45	21·9	...
50	25·4	49·6
55	26·8	48·5
60	26·8	40·2
65	24·8	43·4
70	22·6	69·3

Effect of change in P_H.—Sixteen flasks each contained 15 c.c. starch paste 2 per cent. and 10 c.c. buffer solution (McIlvaine's); the enzyme (5 c.c., 0·6 per cent. solution) was added at 30° and the

reaction allowed to proceed for exactly fifteen minutes, when sugar and precipitable matter were estimated as usual.

No.	P_H	Maltose in 10 c.c. mgms.	Precipitate in 10 c.c. mgms.
1	6·90	9·3	176·1
2	6·50	18·2	173·3
3	6·05	21·7	161·4
4	5·85	22·8	160·6
5	5·70	22·6	169·9
6	5·50	23·2	156·0
7	5·20	24·1	146·1
8	5·00	24·8	139·4
9	4·75	24·1	135·0
10	4·55	23·6	122·9
11	4·40	23·2	115·2
12	4·15	22·8	120·3
13	4·00	20·2	130·0
14	3·80	18·0	122·4
15	3·65	15·3	123·3
16	3·55	12·0	...

Inactivation during dialysis. (*a*) *Malt extract.*—Malt (5 gms.) was shaken with water for an hour, filtered, and the filtrate dialysed in closed collodion bags with running water in constant presence of toluene. After 48 hours the dialysed residue was made up to 100 c.c. and activity tested. Reaction mixtures were composed of 15 c.c. soluble starch solution (2 per cent.), 10 c.c. dialysed enzyme and 5 c.c. water plus salt (0·4408 per cent.). The temperature was 30° and time of reaction 45 minutes and 18 hours; in neither case was any reduction observed.

(*b*) *Precipitated enzyme.*—Malted maize powder (25 gms.) was shaken with water for half an hour and filtered; to the stirred filtrate, which amounted to about 50 c.c., was added 120 c.c. of 95 per cent. alcohol which precipitated the enzyme. This was allowed to settle for three hours and filtered, the residue being shaken with water for half-an-hour and filtered. The filtrate was made up to 50 c.c., 40 c.c. being dialysed in collodion bags in presence of toluene while 10 c. c.

were kept in a tube in the dialyser for the same time. The dialysis was continued in running distilled water when loss of activity was found to be nearly complete on the third day, the product being without effect on starch paste, both alone and in presence of asparagine or sodium chloride.

Search for existence of a dialysable component.—Precipitated enzyme (0·3 gm.) was shaken with water for half-an-hour and filtered, the filtrate being enclosed in a collodion bag and toluene added. Dialysis was carried on in water saturated and covered with toluene, sometimes at room temperature but more frequently in an ice-chest. The liquid was collected at intervals and distilled under reduced pressure at 40–45°, but as this process was too laborious with the volume of liquid involved, concentration by freezing was subsequently adopted and the volume reduced to 100 c. c. After the lapse of five days the enzyme was found to be almost inactive towards starch paste in presence of McIlvaine's buffer solution.

Effect of Salt on the regeneration of inactivated enzyme at different P_H *values.*—The enzyme used in the previous experiment was employed in this series also. Reaction mixtures were made up in six flasks each containing 12·5 c.c. 2 per cent. starch paste to which 10 c.c. of inactivated enzyme was added. The reaction was allowed to continue at 30° for one hour and the sugar then estimated by Amos Peter's method.

P_H	Salt : c.c. of 0·4408 per cent.			Buffer : c. c.	Water : c. c.	Sugar : c. c. of thio-sulphate
6·9	I.	1.	7·5	10	0·0	0·10
		2.	0·0	10	7·5	0·35
5·7	II.	3.	7·5	10	0·0	0·20
		4.	0·0	10	7·5	0·20
4·75	III.	5.	7·5	10	0·0	0·15
		6.	0·0	10	7·5	0·20

The experiment clearly shows that there is no activation by the salt at the different hydrogen-ion concentrations used.

Identification of the sugar produced by hydrolysis.—Starch (20 gms.) was made into a paste with 1000 c.c. of water, cooled and mixed with 0·2 gm. of dry enzyme shaken with 50 c.c. of water. The reaction was conducted at 37° for 42 hours in presence of toluene and then stopped by boiling. After concentration on the water bath to 200 c.c. the

dextrins were precipitated by $2\frac{1}{2}$ vols. of 95 per cent. alcohol, allowed to settle and the supernatant liquid containing the sugar collected and distilled. The precipitate was again dissolved in water, concentrated and precipitated, the supernatant liquid being distilled with the liquid from the first precipitation. The sugar residue was dissolved in methyl alcohol, boiled for half-an-hour with norit and filtered through kieselguhr. Methyl alcohol was removed under reduced pressure and the last traces of moisture absorbed by heating the residue in vacuum over phosphorus pentoxide. The sugar was a white, hygroscopic powder giving $[\alpha]_D + 134°$, and fermentable by *Saccharomyces cereviseae* to the extent of 85 per cent. estimated by decrease in the reducing power. Neither this residue nor the original sugar was hydrolysed by emulsin. These properties indicate maltose.

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II.—ENZYMES FROM THE SEEDS OF *CAESALPINIA BONDUCELLA*.

By Vinayak Narayan Patwardhan.

The seeds of *Cæsalpinia bonducella* commonly known in the vernacular as *Gajga* have a very thick and hard seed-coat which is not easily softened. The decorticated resting seed, containing 15 to 20 per cent. of oil and 2 to 3 per cent. sugar, has been found to contain the following enzymes :—protease, urease, amylase, peroxidase, catalase and oxidases in very small quantity ; it does not contain invertase.

An unexpected result in this examination was that lipase could not be detected. Experiments with seeds containing fat and also with fat-free samples all failed to reveal this enzyme. After germination, which took nearly five weeks, the seed was again tested for lipolytic activity with the same result ; this is difficult to explain in view of the high percentage of oil stored in the cotyledons.

When searching for enzymes in resting seeds the seed-powder was previously extracted with ether for 40 hours, but as lipase sometimes becomes inactivated during extraction the untreated material was also used. In many experiments extracts were made with 20 per cent. glycerol, but this also may act as an inhibiting factor. In special experiments for lipase, therefore, water alone was used and the experiments conducted with shaking, without shaking, with finely emulsified oil and oil temporarily emulsified by shaking. Acetic acid having an accelerating influence on lipase was tried, but without success, and similarly esterase could not be detected, as was shown in experiments with ethyl butyrate. Conclusive proofs of the absence of lipase were obtained by examining the extract, the residue after centrifuging and the top layer of the centrifugate, which according to Hoyer contains most active lipase. Experiments to obtain lipase by extracting the centrifugate with petrol failed to give any results.

The activity of the other enzymes increases during germination.

EXPERIMENTAL.

Extraction of the Enzyme.—Dry fatty or fat-free seed-powder, as the case required, was ground in a mortar with sufficient 20 per cent. glycerol or water to make a loose paste. The mixture after half an hour was centrifuged at 4000–5000 r. p. m. for 10–15 minutes. The supernatant liquid was made up to a known volume, and with addition of a little toluene could be preserved at zero.

Methods used.—The substrates used for protease, urease, amylase and invertase were casein, urea, soluble starch and cane-sugar respectively, the first three giving positive results while inversion was not found in the case of sucrose. All the experiments were conducted at ordinary room temperature except in one or two tests for lipase when the reaction mixtures were incubated for some time at 37°. Tests for peroxidase were made with solutions of *a*-napthol, *p*-phenylenediamine hydrochloride and hydrogen peroxide solutions (indophenol reaction). Catalase was traced by its action on neutral hydrogen peroxide.

A typical example of experiments for investigating the lipase-content is as follows:—The fat-free seed-powder (10 gms.) was extracted with 20 per cent. glycerol, centrifuged and the centrifugate made up to 100 c.c. Reaction mixtures prepared from 5 gms. of oil, 10 c.c. of N/10 acetic acid, 25 c.c. of water and 10 c.c. of (*a*) extract and (*b*) boiled extract, each with a few drops of toluene, were shaken continually in a machine. Portions of 10 c.c. were withdrawn at intervals, 5 c.c. of alcoholic potash added and the mixture titrated with standard sulphuric acid, which showed no change at reaction periods of 19 hrs. 45 mins. and 43 hrs. 15 mins.

The residue was tested for lipase by extracting 10 gms. fat-free seed-powder with water and preparing reaction mixtures from 33 c.c. of oil, 10 c.c. of N/10 acetic acid with 20 c.c. of (*a*) residue, (*b*) the top-layer of centrifugate and (*c*) the remainder of the centrifugate. A few drops of toluene were added to each and the bottles shaken by hand at frequent intervals; 5 c.c. portions were titrated as above and showed no change after 24 hours.

Experiments with fatty seeds extracted with water or with water containing 10–15 c.c. of N/10 acetic acid and conducted on lines similar to above, failed to prove the presence of lipase. In some of the latter experiments the oil was emulsified with gum arabic.

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STUDIES IN THE PROTEINS OF INDIAN FOODSTUFFS.

PART II. The Proteins of the Pigeon Pea (*Cajanus Indicus*).

By P. S. Sundaram, Roland V. Norris and V. Subrahmanyan.

Pigeon pea is a legume raised throughout India and other tropical countries for its seeds, which are used for human consumption, and for its foliage which serves as fodder or as green-manure (Krauss, *Hawaii Agric. Exptl. Stn.*, 1921, Bull. 46; Miller, *J. Agric. Sci.*, 1928, **18**, 569).

Only two varieties of the plant, (1) *Cajanus Indicus bicolor* (arhar) and (2) *Cajanus Indicus flavus* (tur, tuar) are cultivated extensively in India. The former is an annual crop reaching 6–8 ft. and produces light grey seeds which are faintly speckled : the latter is a seasonal, shorter plant and produces small, red seeds. Though their identity in species is still doubted (Watt, *Commercial Products of India*, 1908) yet the varieties are prized equally for their peas, which form the chief protein diet of the people. The peas are prepared by husking after either gentle frying or incipient germination of the seed and used in various ways.

A legume depends for nutritional value largely on the quality of its protein, and since the investigations of Osborne and others have shown that there is a definite relation between the composition of a protein and its nutritional efficiency, an attempt was made to estimate the latter for the *bicolor* and *flavus* varieties by chemical examination of their proteins.

EXPERIMENTAL.

CAJANUS INDICUS BICOLOR (*arhar*).

The seeds, obtained from the Pusa Agricultural Research Institute, were sun-dried, decorticated by being passed through a disintegrator and the cotyledons powdered to a fine meal passing through the 90-mesh sieve. Analysis of the meal gave the following percentages:—Moisture, 8·73; Ash, 3·57; Ether extractives, 2·05; Crude protein (N × 6·25), 22·52; Crude fibre, 3·10; Carbohydrates (by difference), 60·03.

Extraction of albumin and globulins.—To determine the optimum conditions for the extraction of the salt-soluble proteins, trials were

conducted with solutions of sodium chloride and varying (1) concentration of salt, (2) time, (3) temperature of extraction, and (4) reaction of extractant.

1. The meal (10 gms.) passing the 90-mesh sieve was mixed with 250 c. c. of salt solutions of different concentrations, and shaken for one hour at the room temperature, 25°. After filtration, nitrogen in the extracts was determined by the Kjeldahl method, Table I showing the percentages involved.

TABLE I.

Conc. of salt	Nitrogen extracted	Conc. of salt	Nitrogen extracted
0 (water)	46·0	6	60·9
1	51·0	7	59·9
2	58·5	8	59·3
3	62·2	9	58·7
3·5	63·1	10	56·8
4	61·4	15	54·9
5	61·2	20	52·9

Salt solution of 3·5 per cent. was evidently the best suited, the other concentrations extracting less nitrogen.

2. Using a 3·5 per cent. solution of sodium chloride, the periods of extraction were varied, and the corresponding amounts of extracted nitrogen determined (Table II).

TABLE II.

Period of extraction in hours ...	$\frac{1}{4}$	$\frac{1}{2}$	$\frac{3}{4}$	1	2	3	7	14
Percentage of nitrogen extracted ...	62·1	63·1	63·0	63·0	62·8	62·8	62·2	54·5

The maximum amount of protein was extracted in half an hour: the diminution observed on increasing the time was evidently due to partial denaturation of globulins generally observed on continued shaking with salt solution at room temperature.

3. It was observed that 70 per cent. of the total nitrogen was extracted by 3·5 per cent. salt solution on shaking at the room temperature for only 15 minutes and then transferring to the refrigerator (–2°) for 24 hours. Similar extractions carried out six times, successively using fresh portions of saline, yielded 74 per cent.

4. The reaction of an extract prepared as above was acid to litmus, the P_H measured electrometrically with hydrogen electrode being 5·91. Though rendering of the salt suspension neutral to litmus prior to extraction yielded 78 per cent. of protein, addition of alkali was avoided owing to possibilities of (*a*) denaturation and (*b*) racemisation (Dakin, *J. Biol. Chem.*, 1913, **13**, 357) of proteins, isoelectric points of which were far removed from the P_H of the alkali to be used.

Nitrogen extracted by different solvents.—The residue after successive extraction with 3·5 per cent. salt solution at – 2° was treated with boiling 70 per cent. ethyl alcohol and filtered. The residue was then extracted thrice, in the cold, with 0·2 per cent. sodium hydroxide. Determination of nitrogen in different extracts showed percentages passing into the different solvents:—Salt solution, 74·00; alcohol, 0·95; alkali, 21·00.

Distribution of nitrogen in the saline extract.—The extract from 20 gms. of the meal was dialysed in a large parchment cone against distilled water at 5–6° for 10 days when no more salt was found in the dialysate. The precipitated globulins were separated. The supernatant liquid gave no precipitate on saturation with carbon dioxide showing that it contained no globulins. When heated on the water-bath, it began to show turbidity at 43° and yielded a coagulum at 55°. Further heating did not increase coagulation, indicating that probably only one albumin was present. Adding alcohol to the filtrate after removing coagulum gave a minute quantity of white precipitate when the concentration of alcohol reached about 30 per cent.

The precipitates obtained at different stages were separated and their nitrogen contents determined. Percentage distribution of nitrogen in the saline extract was then found to be (1) precipitable by dialysis (globulins), 44·6; (2) coagulable from salt-free dialysate (albumins), 3·75; (3) precipitable with alcohol (proteose), 2·62; (4) other forms, 23·23.

Heat-coagulation.—To determine the number of heat-coagulable fractions in the saline extract, 100 c.c. was treated with 2 drops of 2 per cent. acetic acid, placed in a long thin-walled test tube fitted with thermometer and stirrer, and heated by immersion in a large beaker of water the temperature of which was raised at the rate of 0·5°

per minute. Observations were made with a dark background and diagonal illumination. Turbidity began at 45° and at 52° a coagulum (1) was formed, settling in 2 minutes; the suspension was kept at the same temperature for ½ hour more and filtered. When the filtrate was heated as before opalescence was again noticed at 65°, 78° and 88°, the corresponding coagula (2), (3), and (4) being separated at 70–74°, 80–85° and 94–95° respectively; (2) was small in bulk while (3) and (4) were large.

The experiment was repeated seven times and showed that reproducible coagulations occurred only at 46–55°, 78–85°, and 88 – 95°. Remembering the occurrence of numerous foreign accompaniments to the coagulable proteins, it cannot be stated that the ranges of temperature cited all represented definite proteins in the extract.

Evidence has already been obtained to show that the fraction separating at 46–55° was an albumin. To determine whether the others were globulins, the precipitate obtained on dialysis was washed repeatedly with distilled water, shaken with 3·5 per cent. salt solution, the mixture centrifuged to free it from suspended matter and the temperatures of coagulation noted as already described. It was observed that (1) the major part of the precipitate redissolved in salt solution indicating that it had not altered much in character and (2) opalescence appeared at 78° and 88°, with the corresponding fractions separating at 85° and 95–96°, respectively. It may therefore be inferred that the coagula separating at the higher temperatures were globulins, and that there were two at least.

Preparation of globulin.—Considerable difficulty was experienced in preparing a clear extract of the meal, particularly because of the large amount of sticky matter contained in it. After several attempts the following method was found suitable. The meal (500 gms.) was treated with 2·5 litres of 3·5 per cent. saline and a few c.c. of toluene; after being stirred vigorously for 2 hours by an electrically driven glass stirrer the mixture was allowed to stand in the refrigerator for 24 hours. It was then pulped with scrap filter-paper, and when suitable consistency had been attained was squeezed out through coarse cloth by a hand-press. The extract contained much suspended matter and was passed twice through Sharple's super-centrifuge; on filtering under pressure through thick mats of paper pulp, 2250 c.c. of a clear extract containing 65 per cent. of the total nitrogen was obtained.

Preparation by dialysis.—The extract from 500 gms. of meal was dialysed in parchment cones against distilled water for 15 days.

Globulin, precipitated in large spheroids, was freed from albumin and last traces of chlorides by washing repeatedly with distilled water followed by dilute alcohol. It was dehydrated by standing overnight with absolute alcohol, washing with absolute ether and finally drying in vacuum over sulphuric acid. A crisp white powder was obtained, representing 4·64 per cent. of the meal extracted.

Precipitation by ammonium sulphate.—A similar extract of the meal was saturated with ammonium sulphate by gradually adding the powdered salt with constant stirring, the protein being precipitated slowly in granular flocks and separated by filtration. On shaking with distilled water the precipitate was completely repeptised by the adsorbed salt. This extract was filtered through paper mat and dialysed in parchment cones until free from salt. The precipitated globulin was washed free from albumin, dehydrated and dried in the manner described already, forming a white powder resembling that obtained by dialysis and representing 4·8 per cent. of the meal.

Analysis of the globulin preparations.—The two preparations were dried at 110° and analysed for their constituent elements (Table III). Carbon and hydrogen were determined by combustion: sulphur by Hoffman and Gortner's modification (*J. Amer. Chem. Soc.*, **45**, 1033) of the original method of Benedict and Denis: and nitrogen by the Kjeldahl process.

TABLE III.

Percentages on ash-free basis.

	C	H	N	S	O	P
By dialysis (ash, 0·52 per cent.)	52·90	6·91	15·94	0·46	23·79	Nil
By precipitation (ash, 0·42 per cent.)	52·78	6·84	16·01	0·45	23·92	Nil

Differences being within the range of experimental error, it may be inferred that the two preparations were identical in their elementary composition.

Fractional precipitation.—With a view to dividing the globulins into several fractions by differential precipitation with ammonium sulphate, trials were carried out with the saline extract from 500 gms. of the meal by adding to it powdered ammonium sulphate so as to raise the concentration of salt gradually from 10 to 100 per cent.

saturation. Precipitation began at 30–40 per cent. saturation, the protein separating in 15 minutes. On filtering and adding more salt, increasing amounts of protein separated at different stages up to 90 per cent. saturation. The different fractions thus obtained were separated, washed with ammonium sulphate of the same concentration, redissolved and precipitated by dialysis. They were then dehydrated, dried and examined for the following factors, (1) yield of moisture-free protein, (2) percentage of sulphur and (3) response to glyoxylic acid test for tryptophan (Hopkins and Cole). The same fractions were obtained from another portion of extract, redissolved in 3·5 per cent. saline, and the corresponding temperatures of coagulation determined. The different observations are recorded in Table IV.

TABLE IV.

Percentage saturation	Yield in grams	Percentage of sulphur	Tryptophan reaction	Solubility in saline	Coagulation temperature
0–40	3·1	0·99	Strong	Sparing	95° on prolonged heating
40–50	2·5	0·58	Faint	Partial	86° and again 95°
50–60	2·1	0.45	,,	Complete	85—88°
60–70	5·9	0·39	Very faint	,,	,,
70–80	5·8	0·38	,,	,,	,,
80–90	5·4	0·39	,,	,,	,,

It may be inferred from the above that the fractions contained at least two globulins, one precipitated between 0 and 40 per cent. saturation, coagulating at 95°, rich in sulphur and tryptophan and the other precipitated between 60 and 90 per cent. saturations, coagulating at 85–88°, poor in sulphur and tryptophan. Since the previous coagulation experiments also gave evidence of the presence of only two distinct globulins, it is probable that the fractions separating between 40 and 60 per cent. saturation were varying mixtures of the two, as indicated by their intermediate sulphur contents.

Refractionation.—To obtain pure specimens, refractionation of the portions separating between the two definite ranges was attempted, constancy of sulphur-content being taken as evidence of purity. It was observed that the fraction separating between 0 and 40 per cent. saturation was already denatured and could not be redissolved in saline. The one separating between 60 and 90 per cent. saturation was however readily peptised and on refractionation was found to contain 0·37 per cent. of sulphur. This being nearly identical with

those obtained for the preparations by single fractionation (Table IV) it may be inferred that the latter was pure.

Preparation of globulins I *and* II.—Globulin I separated in flocks on adding to a clear salt extract of the meal solid ammonium sulphate to 40 per cent. saturation. The precipitate was collected on a paper filter and washed repeatedly with 40 per cent. ammonium sulphate. It was then repeptised by the adsorbed salt on shaking with distilled water and dialysed in parchment cones against distilled water until free from sulphate; the precipitated protein was washed successively with distilled water and dilute alcohol, and dehydrated by washing with absolute alcohol followed by ether. The yield was 0·65–0·80 per cent. of the meal.

In the filtrate from globulin I the saturation of ammonium sulphate was increased to 60 per cent. After removing the precipitate of mixed globulins the saturation of ammonium sulphate was raised to 90 per cent. when globulin II separated. The precipitate was repeptised, filtered, dialysed free from salts, and dehydrated as already described for globulin I. The yield was 3·2–3·5 per cent. of the meal.

Composition of globulins I *and* II.—Three independent preparations (A, B and C) of each globulin were made and their individual composition determined (Table V).

TABLE V.

Percentages on ash-free basis.

	Globulin I				Globulin II			
	A	B	C	Average	A	B	C	Average
C ...	53·17	53·84	53·65	53·55	52·41	52·84	51·98	52·41
H ...	6·92	7·16	6·99	7·02	6·88	6·98	6·84	6·90
S ...	0·99	1·01	1·04	1·01	0·37	0·39	0·39	0·38
N ...	15·21	15·01	15·34	15·19	16·22	16·10	16·25	16·19
O ...	23·71	22·98	22·96	23·23	24·12	23·69	25·54	24·12

The percentage of ash in the preparations was as follows:—Globulin I: *A*, *0·64*; *B*, *0·72*; C, *0·82*. Globulin II: A, *0·13*; B, *0·14*; C, 0·24.

Cajanin and Concajanin.—The foregoing observations indicate that globulins I and II have definite composition and are distinct from each other in their percentage of sulphur and nitrogen. In accordance with convention, globulin II which was present in the larger quantity and was thus the principal protein of the seed was named *cajanin* after the genus, and globulin I which accompanied it, *concajanin.*

Cajanin when dried is snow-white, dissolves freely in alkalis, but less readily in acids; it dissolves sparingly in neutral solvents although before treatment with alcohol and ether the globulin was freely soluble. It gave all the general reactions for proteins but only feeble response to Hopkins and Cole's and Molisch's tests. The isoelectric point as determined by the method of Csonka, Murphy and Jones (*J. Amer. Chem. Soc.*, 1926, **48**, 763) using the acetate buffer is P_H 4·95.

Concajanin, is a grey powder resembling cajanin in properties except that it (1) was sparingly soluble in neutral solvents even before dehydration with alcohol and ether and (2) responds to the tests for tryptophan and carbohydrate. The isoelectric point approximates to that of cajanin being P_H 5·22.

Protein extracted by alkali.—To identify the protein left after exhaustive salt extraction, the residue from 500 gms. of meal was extracted with 0·2 per cent. sodium hydroxide and a clear filtrate obtained on passing the suspension through paper pulp. The protein in the extract was precipitated on rendering slightly acid to litmus. It was filtered, redissolved in alkali, again precipitated with acid, filtered, washed and dried. A pale-red powder representing 0·9 per cent. of the meal and containing 0·52 per cent. of ash was obtained. Its ash-free composition was C, 52·94; H, 6·98; S, 0·41; N, 15·86 and O, 23·81 per cent.

The isoelectric point of this protein was P_H 5·0. It was precipitated from alkaline solution only on adding ammonium sulphate up to at least 40–50 per cent. saturation. The properties correspond more with those of the globulins already described than with those of typical glutelins, which are characterised by (1) precipitation from alkaline solutions by ammonium sulphate even at 1 to 18 per cent. saturation and (2) isoelectric points approximating to P_H 6·5 (Csonka and Jones, *J. Biol. Chem.*, 1927, **73**, 321). In view of the foregoing considerations it may be inferred that the protein extracted by alkali was merely the seed-globulin not fully extracted by salt solution, but readily peptised by dilute alkali. Calculated on the basis of its sulphur-content it appears to have contained cajanin to the extent of 95·2 per cent.

A revised estimate of the approximate percentage distribution of nitrogen in the pea would therefore be :—albumin, 3·8 ; cajanin, 57·2 ; concajanin, 8·4 ; proteose, 2·6 ; other forms, 28·0.

Albumin.—The meal (500 gms.) was extracted with distilled water and the clear extract (2 litres) dialysed for 10 days at 5–6° against distilled water, when the dialysate gave only faint opalescence with silver nitrate. After separation from globulins, the extract containing albumin was saturated with carbon dioxide to precipitate any residual globulin and the acidified filtrate heated at 55° for half-an-hour. The small coagula thus obtained from several extracts were collected, washed with water and alcohol, dehydrated and dried, yielding 4 gms. of a pale grey substance representing 0·4 per cent. of the meal extracted and containing 0·45 per cent. of ash.

The isoelectric point of the albumin was P_H 4·2, and the composition (ash-free) :—C, 54·82 ; H, 6·84 ; N, 14·80 ; S, 1·18 ; O, 22·36 per cent.

Nitrogen distribution.—About 3 gms. each of cajanin, concajanin and the albumin were used for determining the distribution of nitrogen by the Van Slyke method as improved by Plimmer and Rosedale (*Biochem. J.*, 1925, **19**, 1004) with the following modifications : (1) after precipitation with phosphotungstic acid the diamino-fraction was separated by centrifuging and (2) arginine nitrogen was determined by Kœhler's method (*J. Biol. Chem.*, 1920, **42**, 267). Sulphur in the diamino-fraction was determined by the method of Plimmer and Lowndes (*Biochem. J.*, 1927, **21**, 247).

Cystine, tryptophan and tyrosine.—The cystine figures obtained by the Van Slyke method cannot be taken as correct. Being readily racemised during the hydrolysis, it should have been incompletely precipitated, the phosphotungstate of the racemised acid being more soluble than that of the optically active one. Independent determinations were therefore carried out in the acid hydrolysate of the proteins by the methods of (1) Folin and Looney (*J. Biol. Chem.*, 1922, **51**, 421) making use of sodium hydroxide instead of sodium carbonate as modified by Hunter and Eagles (*J. Biol. Chem.*, 1927, **72**, 177) and (2) Sullivan (*J. Biol. Chem.*, 1927, **74**, xiv).

Tryptophan was determined by the methods of (1) Komm (*Z. Physiol. Chem.*, 1926, **156**, 202) using *p*-dimethylaminobenzaldehyde, and (2) Tillmans and Alt (*Biochem. Zeit.*, 1925, **164**, 135), using in both cases a solution of pure tryptophan as standard. It was however found difficult to compare colours by Komm's method, those developed by the proteins having always a shade of violet different from that

TABLE VI.

Distribution of Nitrogen in Concajanin, Cajanin and Albumin (Percentages of total nitrogen).

Form of Nitrogen	Concajanin I	Concajanin II	Concajanin Average	Cajanin I	Cajanin II	Cajanin Average	Albumin
Amide ...	9·05	8·97	9·01	10·42	10·20	10·31	9·05
Humin :— ...	2·0	1·93	1·98	1·22	1·17	1·20	3·13
Insoluble ...	1·15	1·07	1·11	0·75	0·66	0·71	1·61
Soluble (adsorbed by lime)	0·88	0·86	0·87	0·47	0·51	0·49	1·52
Basic :— ...	29·43	28·51	28·97	24·45	25·31	24·88	24·96
Arginine ..	14·11	14·21	14·16	11·34	11·40	11·37	12·04
Cystine ...	1·01	1·23	1·12	0·28	0·34	0·31	1·31
Histidine ...	4·09	3·53	3·81	4·52	5·48	5·00	4·43
Lysine ...	10·22	9·54	9·88	8·31	8·09	8·20	7·18
Non-basic :— ...	59·42	59·88	59·65	64·81	63·33	64·07	62·84
Amino ...	55·64	57·22	56·43	62·69	61·65	62·17	59·26
Non-amino ...	3·78	2·66	3·22	2·12	1·68	1·90	3·58
Total ...	99·93	99·29	99·61	100·90	100·01	100·46	99·98

given by tryptophan. Since similar difficulties were also experienced in trials using egg and blood albumins the results obtained by Komm's method were not recorded. The method of Tillmans and Alt worked satisfactorily.

Tyrosine was estimated independently by the method of Folin and Ciocalteu (*J. Biol. Chem.*, 1927, **73**, 627) and together with tryptophan by that of Tillmans, Hirsch and Stoppel (*Biochem. Zeit.*, 1928, **198**, 379). The percentages of the essential amino-acids as determined by the different methods are given in Table VII.

TABLE VII.

Amino-acid	Albumin	Concajanin	Cajanin	Method
Lysine ...	5·54	7·90	6·93	Van Slyke
Arginine ...	5·53	6·75	5·72	,,
Histidine ...	2·42	2·16	2·99	,,
Cystine ...	2·73	2·24	1·41	Folin and Looney
,, ...	2·32	1·98	1·39	Sullivan
Tryptophan ...	2·05	1·48	0·21	Tillmans and Alt
,, ...	...	1·53	0·12	Tillmans, Hirsch and Stoppel
Tyrosine ...	...	3·45	3·16	Tillmans, Hirsch and Stoppel
,, ...	...	3·61	3·29	Folin and Ciocalteu

The figures for cystine by Sullivan's method are probably more trustworthy than those by that of Folin and Looney because the latter method is not specific for cystine (U. du Vigneaud, *J. Biol. Chem.*, 1927, **75**, 393). The methods of Tillmans and Alt for tryptophan and Folin and Ciocalteu for tyrosine gave more consistent results than the combined one of Tillmans, Hirsch and Stoppel; the figures obtained by the first two have therefore been taken as correct.

CAJANUS INDICUS FLAVUS (*tur tuar*)

The seeds of the above variety were from Thiruppattur in South India. The meal was prepared in the manner described already and on analysis was found to have the following percentage composition :— Moisture, 7·07 ; Ash, 3·57; Crude fat, 0·83 ; Protein (N × 6·25), 24·09 ; Crude fibre, 4·21 ; Carbohydrate (by difference) 60·24.

Saline 3·5 per cent. extracted 78·0 per cent. of the total nitrogen at $-2°$ and yielded on fractional precipitation with ammonium sulphate followed by repeptisation and dialysis in the manner described already the two globulins concajanin and cajanin. Pure specimens of the proteins were analysed for their sulphur and tryptophan content (Table VIII).

TABLE VIII.

Constituent and method	Percentage	
	Cajanin	Concajanin
Sulphur (Hoffman and Gortner, *loc. cit.*) ...	0·40	1·11
Tryptophan (Tillmans and Alt, *loc. cit.*) ...	0·32	1·61

The results approximate to those from peas of the *bicolor* variety.

DISCUSSION.

As observed in other plants of the natural order *leguminosæ*, the seeds of *Cajanus Indicus* contain among their reserve proteins two globulins which (1) appear to be characteristic of genera and independent of differences in species or varieties, (2) form the chief proteins of the seed and (3) differ from each other with regard to their sulphur contents and limits of precipitability with ammonium sulphate (compare Jones and Johns, *J. Biol. Chem.*, 1916–17, **28**, 67; Jones, Gersdorff, Johns and Finks, *J. Biol. Chem.*, 1923, **53**, 231).

The three proteins differ from each other in their general distribution of nitrogen, particularly in their amide and basic amino-acid contents. Cystine and tryptophan decrease in the order, albumin, concajanin and cajanin: lysine and arginine also vary, but follow a different order. Cystine appears to be the main sulphur-containing amino-acid in cajanin, while in concajanin and the albumin there appears to be also a large proportion of another sulphur-containing amino-acid of a lower molecular weight. The presence of identical proteins containing similar amounts of sulphur and tryptophan suggests that the peas of the *bicolor* and the *flavus* varieties are of almost equal nutritive value.

All the proteins in the pea are rich in tyrosine and contain the requisite amounts of cystine and the other essential diamino-acids;

but although the albumin and concajanin contain the necessary amounts of tryptophan, cajanin, the chief protein of the seed and representing over 58 per cent. of the total nitrogen, is deficient in that acid which is essential to growth and maintenance. Although this tends to reduce, to some extent, the independent nutritive quality of the pea, it does not lower its value in a mixed diet containing the essential amino-acids generally supplied by a variety of vegetable and animal proteins, so that deficiency in one is made up by adequate supply from another. In India where the peas are generally taken together with cereals it ought to be of high supplementary nutritive value, since it supplies useful amounts of the essential diamino-acids, particularly lysine, in which the latter are generally deficient.

SUMMARY.

1. The chief proteins of the peas of *Cajanus Indicus* of both the *bicolor* and *flavus* varieties were two globulins, cajanin and concajanin which (*a*) accounted for about 58 and 8 per cent. respectively of the total nitrogen and (*b*) differed from each other in their sulphur and tryptophan content. An albumin representing about 4 per cent. of the total nitrogen was also prepared and examined.

2. The three proteins contained requisite amounts of cystine, arginine and lysine: but since cajanin the chief protein of the seed was found to be deficient in tryptophan, the pea could not be said to make a complete protein food by itself. It should, however, make a valuable supplement to the cereal foods which are generally deficient in the essential diamino-acids.

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THE ELECTRICAL CONDUCTIVITY OF THIN OIL FILMS.

Part I.—General Nature of the Phenomenon.[1]

By H. E. Watson and A. S. Menon.

It has long been a common practice to cover accumulator terminals with vaseline; Price and others in a discussion at the Physical Society (*Proc. Phys. Soc.*, 1903, **18**, 479) mentioned that potentiometer contacts were improved by immersion in kerosene oil; Manley (*Phil. Mag.*, 1917, **33**, 211) found that the resistance of the plugs in a resistance box was invariably more constant and very nearly always lower when they were lubricated with vaseline than when they were dry; Kraus (*Electrotechnik u. Maschinenbau*, 1920, **30**, 1) and Melsom and Booth (*J. Inst. Elec. Eng.*, 1922, **60**, 889) in investigations upon contact resistances, such as switches for electrical machinery, obtained similar results. Vaseline and heavy paraffin oil are now widely used for application to plugs and sliding contacts on measuring instruments.

In view of the fact that these substances are ordinarily regarded as excellent insulators, their use for the above purpose appears somewhat anomalous, and the object of the present investigation is a detailed examination of the region in which a transition from insulation to conduction appears to take place.

Branly (*Compt. rend.*, 1912, **155**, 933), in his experiments upon the coherer, found that films of guttapercha, collodion, mica, celluloid and paraffin wax from 5 to 25 μ in thickness conducted electricity when pressed between optically polished metal discs, and the resistance was reduced by an oscillatory discharge in the vicinity. He did not measure the actual thickness of the dielectrics. Wilson and Daniel (*Ind. Eng. Chem.*, 1922, **14**, 683) measured the contact resistance of two flat metallic surfaces pressed together and found that a dry resistance of 0·03 ohm increased on immersing the surfaces in mineral oil, continued to increase for about 6 hours and reached a final value of 4·4 ohms, pointing to the gradual building up of an adsorbed film. On application of a very small 1,000 cycle alternating E.M.F. the resistance fell rapidly to 0·4 ohm. These results do not appear to be in agreement with those of previous workers since the resistances

[1] Reprinted from the *Proceedings of the Royal Society*, 1929, A **123**, 185.

are much higher. The resistance of the plugs measured by Manley was of the order of 0·002 ohm. The difference may be due to the pressure, which in Wilson and Daniel's experiments was 311 gm./sq. cm., while in the other cases it was probably much greater.

The present paper deals more with the phenomena of breakdown than with the actual values of resistance when 'contact' has been established, and is largely an account of the experimental methods used for determining the nature and the thickness of films obtained in different ways. For most of the work a heavy paraffin oil $d30° = 0·886$ and with very slight action on bromine has been used. The breakdown strength, kindly measured by Mr. Yoganandam in the Institute high tension testing laboratory, was found to be 92,000 volts r.m.s. per centimetre between 2·5 cm. discs 3·8 and 1·2 mm. apart at 60 cycles. Although suffering from the disadvantage of being a mixture of unknown composition, yet, for preliminary work, this is more than counterbalanced by the high insulating properties, large viscosity, low volatility and freedom from action upon metals of the oil, coupled with the fact that it is readily obtainable in large quantities while, since it is actually used for lubrication of contacts, an investigation of its electrical properties is desirable.

It is found that there is a critical thickness of the order 10μ at which the dielectric strength falls very suddenly and that breakdown takes place in two stages. Measurements have also been made upon the air film which is formed under certain conditions between two plane surfaces.

EXPERIMENTAL.

The experiments were started shortly after the publication of Hardy and Bircumshaw's Bakerian Lecture (*Proc. Roy. Soc.*, 1925, A **108**, 1) on boundary lubrication in which the coefficient of friction was shown to vary with the thickness of the oil film. It was thought that these results might be of value in correlating the thickness of the film with the conductivity, and consequently an apparatus somewhat similar to the one used by these authors was set up and friction measurements were made simultaneously with the others.

The determination of the capacity of the condenser formed by the two metal surfaces and the oil film suggested itself as a possible method of measuring the thickness of the latter and, consequently, arrangements were made to measure this quantity. The whole apparatus is shown diagrammatically in fig. 1.

The slider A weighed 30·3 gm. and consisted of a triangular 6 mm.

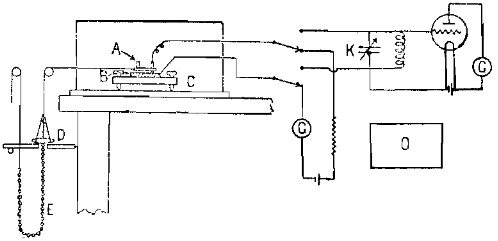

FIG. 1.

plate of 35 mm. side with three 6 mm. legs. These were turned down to 3 mm. at the ends, case-hardened and polished. They rested upon a polished steel plate B, half of which was silvered, supported upon a levelling platform C. The whole could be enclosed, if required, by a glass cover with ground edges under which was a dish of phosphorus pentoxide, but the presence of this drying agent had no marked effect, probably because experiments were not made with perfectly clean surfaces and the laboratory air was very pure and, at the time of the experiments, exceedingly dry. For determining the friction, a fine thread from A passed over a pulley and supported a scale pan D with an adjustable release which would only allow a drop of about 1 mm. Weights nearly sufficient to move A were placed in the pan and the final adjustment to the weight necessary to cause slip was made by means of a fine calibrated chain suspended below D, the principle being that of the chainometric balance. In this way the weight could be increased very smoothly and measurements could be rapidly repeated.

In order to determine if the film was conducting, the slider and plate could be connected in series with a battery, galvanometer and high resistance (about 100,000 ohms) to limit the amount of current passing, connection being made to the slider by a very fine wire to avoid strains. The capacity of the condenser when the film was non-conducting was measured by connecting it in parallel with a calibrated condenser K and an inductance. The circuit could be tuned to resonance with the oscillations from a separate oscillator O, the resonance point being determined by means of the simple valve circuit shown (*Cf.* Hobbs, *Wireless World*, 1924, **14**, 77). Wave-lengths varying from 100 to 300 m. were used. In order to avoid errors due to change in lead capacity, the circuit was first set to resonance with A

resting on the oil film, the slider was then raised about 3 mm. by inserting a small ebonite strip between the top and the plate B and measuring the change in K necessary to restore resonance. This change was substantially equal to the capacity of the condensers formed by the three legs of the slider and the plate when separated by the oil film.

WIPED FILMS.

As soon as the experiments were started, it was found impossible to obtain a non-conducting film by placing oil, vaseline, or even a thin film of a solid, on the plate and standing the slider upon it, but if the substance was wiped off until almost invisible, a very stable film was obtained which would frequently withstand the application of 100 volts. The nature of this type of film will be discussed later.

Preliminary experiments were made with myristic acid. A small crystal was melted on the plate, previously thoroughly cleaned by boiling in alcohol and allowed to cool in a vacuum desiccator. The film so formed was wiped, and, when the slider was placed on it, was non-conducting. A number of measurements of the coefficient of friction were made, the slider being allowed to move about 1 mm. and then replaced in its original position.

TABLE I.

Coefficient of Friction, Steel-Silver, Myristic Acid, Non-conducting.

	a			*b*	
Weight:	30·3	80·7	130·8	30·3	130·8
Coefficient of friction	0·413	0·283	0·253	0·374	0·185
	0·390	0·279	0·252	0·354	0·184
	0·400	0·282	0·249	0·354	0·183
	0·403	0·284	0·237	0 337	0·187
		0·282	0·255	0·347	
		0·284	0·250		
Mean...............	0·401	0·282	0·249	0·353	0·185

The values (*a*) and (*b*) were obtained with different films, and it will be observed that, while sufficiently concordant results with a maximum variation of about 10 per cent. can be obtained in the same part of the same film, much larger variations are found with different films. In the case of a brass plate and the steel slider, a mean value of 0·355 was found for a load of 30·3 gm. The area of contact of each leg of the slider was 6·75 sq. mm., so that the actual loads in the three cases were approximately 150, 400 and 650 gm./sq. cm.

Measurements were next made of the thickness of the films by the dielectric constant method. It was at once found that the film thickness was by no means of molecular dimensions, but large even in comparison with the wave-length of light. Very exact optical polishing of the surfaces was consequently not necessary. The first slider used was slightly convex at the ends of the legs, the flatness over about one-third of the total area being within 1 μ. This was measured by placing a piece of optically worked glass over the legs and observing the positions of the Newton's rings. These were drawn on a larger scale on squared paper and the area corresponding with each thickness computed. The approximate thickness of the film was calculated by means of a curve showing the relation between minimum thickness and capacity.

Table II gives some results for this series of experiments with different films.

A number of experiments with myristic acid showed that widely divergent values could be obtained by placing the slider on different films or on different parts of the same film. The first two figures are the maximum and minimum capacities which were found in this way. The coefficients of friction varied between 0·37 and 0·44, but there appeared to be no relation between the coefficient of friction and the capacity. For determining the variation with pressure, the same part of the same film was used, and it may be seen that the coefficient of friction ρ decreases considerably with increase of pressure in agreement with Hardy's results (*loc. cit.*), but the capacity changes very slightly except in the case of paraffin wax. These results are shown in brackets. Where two sets are given for the same substance, the figures refer to different films or the same film after wiping. The various substances used were not specially purified for the preliminary experiments as the object was to ascertain the magnitude of the effect of the material of the film upon the thickness. Actually, the variations with one substance were so great that it was impossible to compare the results for different substances, although it is interesting to note that the thickest films were obtained with paraffin oil and

TABLE II.

Coefficient of Friction and Minimum Thickness of Non-conducting Wiped Films.

Film	Weight, gm.	ρ	Capacity, $\mu\mu$ F	Thickness, mm $^{-8}$ ($\epsilon = 1$)
Myristic acid, silver-steel	30·3	0·43	50·8	1·27
	30·3	0·40	34·2	2·42
	30·3	0·45	36·0	2·24
	60·3	0·31	37·0	2·15
	130·3	0·24	38·9	1·99
	230·3	0·25	39·5	1·93
Paraffin wax, silver-steel	30·3	0·36	29·4	3·10
	80·3	0·175	32·4	2·62
	130·3	0·175	38·0	2·07
	230·3	0·135	44·0	1·63
Paraffin oil, steel-steel	30·3	0·39	22·5	4·55
	80·3	0·19	23·5	4·30
	130·3	0·21	26·4	3·67
	30·3	0·39	45·7	1·52
	80·3	0·32	47·5	1·43
Stearic acid, steel-steel	30·3	0·33	52·0	1·20
	80·3	0·195	57·0	1·03
	130·3	0·155	60·8	0·91
Oleic acid, steel-steel..................	30·3	0·40	47·5	1·43
	80·3	0·26	48·0	1·41
	30·3	0·36	31·0	2·85
	80·3	0·27	31·0	2·85
Linolenic acid, steel-steel............	30·3	0·44	51·5	1·25
	80·3	0·34	53·5	1·16

the thinnest with stearic acid. The thicknesses given are the minimum thicknesses, calculated in the manner already described, but, apart from the uncertainty introduced by the curvature of the slider legs, they involve the assumption that the film under each leg is of equal thickness and, consequently, cannot be considered as accurate values. Subsequent experiments after regrinding and polishing the legs until they were almost flat gave very similar results, so that the error is probably not large. In any case the figures for the variation in thickness on weighting are likely to be substantially correct.

The thickness has been calculated with the value 1 for the dielectric constant of the film, whereas the value for all the materials used was not far from 2. The original calculations were

made with the latter figure leading to double the film thicknesses given. It was, however, quite obvious that these results were impossible as a film even 1 μ in thickness was quite opaque, while the wiped films were hardly visible. On discussing the matter with Sir William Hardy, he suggested that the films really consisted of air. This would account for the high insulating properties, since it has been shown by Broxon (*Phys. Rev.*, 1922, **20**, 476) that air films of the order of 0·5 μ in thickness will withstand a gradient of 640,000 volts per centimetre before breaking down.

Examination under the microscope revealed the rather unexpected fact that the films are not continuous, but consist of a number of minute globules which appear not to wet the surface. Fig. 2 is a microphotograph of a film of paraffin oil on stainless steel in the initial stages of wiping. Fig. 3 is an adjacent portion of the steel plate upon which a

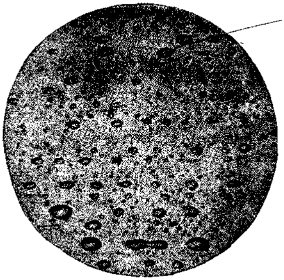

Fig. 2.

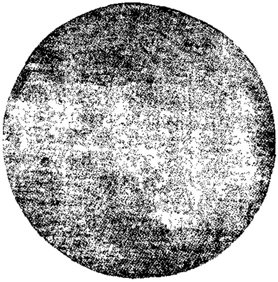

Fig. 3

× 80

steel cylinder had stood overnight in a pool of the oil. Both portions were wiped simultaneously and had stood for 24 hours before being photographed.

The appearance is quite different and it is evident that wetting of some kind has taken place. Melted myristic and oleic acids also formed globules, but nonyl alcohol spread in a continuous thin layer.

These facts suggest that non-conducting wiped films consist of a number of small oil globules between two adsorbed films of air or water vapour on the metallic surfaces, but their exact nature seems to be one of some complexity, and further discussion will be deferred until the experiments with flooded films have been described.

Flooded Films.

As already mentioned, it was not found possible to obtain a stable non-conducting film when the slider was placed in a pool of lubricant. This was also the case when a steel cylinder of diameter 10·9 mm. and weighing 11·2 gm. was substituted for the slider. It was subsequently found that discs of brass and case-hardened mild steel 18 mm. in diameter and weighing 8·6 and 7·9 gm., respectively, would not only float on the oil but would remain suspended above the plate when the oil was removed.

In order to obtain a film of varying thickness which changed slowly enough for observations to be made with some accuracy, the following device was adopted.

A piece of thick plate glass A, fig. 4, with a good optical surface, was silvered or platinised by splashing for half its width, as shown by the shaded area. The silvering was removed for 1 cm. at one end. The glass was placed on a stainless steel mirror or a similar piece of glass with the unsilvered end in contact and the other supported on a small piece of mica C, 32 μ thick, cut into two. The silvering thus formed a wedge with the mirror but was insulated from it. Electrical contact was made at the end of the plate and a drop of liquid was allowed to flow between the two pieces of mica. This gradually extended along the wedge and its progress could be watched through the clear side of the glass. At a certain point the film became conducting and the wedge was then opened and the farthest point to which the liquid had penetrated was observed. From this the thickness could be deduced. The thickness of the mica was measured as accurately as possible with a micrometer. Capacity measurements were not very satisfactory, possibly owing to irregularities in the silvering. The values obtained for the thickness were greater than by direct measurement.

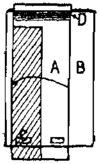

Fig. 4.

It was found that with very thin layers of oil between two slightly curved plates there was a considerable attraction which was sufficient to bend the plates. In the present series of experiments a few interference bands could be seen at the end of the plate at D, and these did not move appreciably on admitting oil, so that mechanical distortion was absent.

The surfaces were carefully cleaned in boiling ethyl alcohol or by washing with petrol followed by anhydrous methyl alcohol, and paraffin

oil or triolein run into the wedge. Some of the individual results for the thickness at which the film became conducting were as follows :—

Paraffin oil—

Silver-steel	A.C., 8 μ, 15 μ.
Silver-silver	D.C., 6 v.–10 μ ; 110 v.–11 μ.
*Platinum-steel	D.C., 2 v.–11 μ ; 24 v.–11μ ; 80 v.–13 μ.
Silver-steel	D.C., 2 v.–14 μ.

Triolein—

Silver-silver........	D.C., 6 v.–13, 16 μ ; 110 v.–18 μ.
Silver-steel	D.C., 6 v.–13, 13 μ.

In all cases the point of breakdown was very sharply defined. The thicknesses are not very exact owing to the difficulty of determining the effective thickness of the mica, but the same mica was used throughout so that the values are comparable. In the experiment marked with a star, a quartz fibre 27 μ in diameter was used instead of the mica and the diameter of this could be measured with some accuracy. The silver films softened after a few experiments and had to be frequently renewed, but the platinum resisted the oil better.

The most striking feature of these results is that the breakdown thickness appears to be nearly independent of the applied voltage. Exact reproduction of the experimental conditions was a matter of some difficulty, but a good example is seen in the three experiments at 2, 24 and 80 volts with platinised glass. These were made as far as possible under comparable conditions and conduction occurred at very nearly the same thickness in all cases. The possibility of the result being due to a small projecting piece of metal was excluded by the fact that the oil ran rather unevenly, and although at the same distance from the end, it was in contact with different parts of the film when breakdown occurred. The figures for triolein appear to be slightly larger than those for paraffin oil. In most of these experiments only the first stage of conduction was obtained ; that is to say, the film resistance fell to about 1 megohm.

Although the wedge method was of value for determining the approximate thickness at which conduction took place, its application was limited and it seemed desirable to supplement the results in other ways. For this purpose a more accurate method of measuring capacities was adopted similar to the one used by one of us (*Proc. Roy. Soc.*, 1927, A **117**, 43) for determining dielectric constants. The condenser under observation was placed in parallel with a precision

condenser and the capacity determined by the method of beats at a frequency of about 600 k.c. Approximate measurements of the resistance of the condenser could be made simultaneously. With this arrangement a vertical displacement of 0·003 μ could be readily measured, and it was possible to determine if necessary much smaller shifts by means of a vernier condenser. The method possessed the advantage that even rapid movements could be qualitatively followed by ear.

Attempts were first made to determine the thickness at which conduction took place by placing the slider, or the steel cylinder already mentioned, in a pool of oil and noting the maximum capacity reached before oscillation stopped. With paraffin oil, conduction occurred within about 2 seconds and no reliable measurements were possible. With the cylinder, the thinnest film measured was 10 μ in thickness and with the slider and vaseline 8 μ, but these values are probably too large. The magnitude of the alternating E. M. F. applied could not be measured as it varied rapidly, but it was certainly less than 2 volts. The load per square centimetre in the two cases was 144 and 12 gm. When brass and steel discs corresponding to loads of 3·6 and 3·3 gm. per square centimetre were used, they floated in the oil and the films did not conduct. Another system was then tried. A condenser was made by standing the steel cylinder on two quartz or pyrex fibres stretched across a stainless steel mirror. For a very convenient method of drawing the glass fibres we are much indebted to Dr. E. P. Metcalfe, of the Central College, Bangalore. A small bead of glass is melted on an electrically heated platinum wire and the fibre drawn by touching the bead with a very fine platinum wire and pulling out by hand. By regulating the temperature and the rate of pulling, a fibre of approximately correct diameter can be drawn.

Numerous observations have been made with condensers of this type using the method of beats. The results are somewhat irregular, but certain features appear to be fairly definite. The thickness of the air film calculated from the capacity is always slightly greater than the measured thickness of the fibres except in one or two cases in which a fibre may have been displaced while putting down the cylinder. The difference is more noticeable for the thinner fibres. For film thickness of the order of 8 μ and under, it was frequently noticed that the cylinder at first assumed a metastable position and could be made to sink by gently tapping the plate or cylinder, but even prolonged tapping would not reduce the thickness to that of the fibres. At the same time attempts to remove a fibre only result in fracture, so that adhesion of some kind is present. The thicker films will stand a D.C. voltage of over 100 without breaking down, the insulation resistance being over

50 megohms and potential gradients of 100,000 v/cm. having been reached. The thinner films break down frequently on application of from 2 to 40 volts and become partially conducting, sometimes the resistance becomes very low. Usually, but not always, a film which has broken down subsequently conducts on application of a lower E.M.F., although it did not do so previously. Tapping restores the insulation. This might possibly be due to a thin bridge of metal, but a calculation of its diameter from the measured resistance in one or two cases assuming the ordinary resistance law to hold, gives a diameter of the order of 3×10^{-7} cm., a rather unlikely quantity. The alternating voltage applied to the film varied from 2·5 to 3 volts r.m.s., but it was not convenient to determine the breakdown point for alternating current with the more sensitive apparatus, as the power factor of the thinner films was appreciable and tended to stop the oscillations before actual conduction occurred. It is noteworthy that all films which would withstand 2 volts D.C. could be measured with the alternating E.M.F. mentioned, a result not to be anticipated from the work of other observers.

In making these measurements the cylinder was placed on the fibres and the capacity of the system measured. Any change on tapping was noted and oil was then run under the cylinder and the capacity remeasured. The values for the dielectric constant were very close to the correct value and afford a remarkable confirmation of Sir William Hardy and Miss Nottage's observations (*Proc. Roy. Soc.*, 1928, A **118**, 224), which reached India just after these experiments had been conducted, that a flat steel cylinder does not come into contact with a flat plate when stood upon it, but that a stable air film intervenes and the distance between plate and cylinder is not affected by introducing a lubricant between them. The examples in Table III show, however, that this is not true if the system is in the metastable state already mentioned, since in this case high values are obtained for the dielectric constant indicating that the lubricant pulls the cylinder down to the stable distance. It may be mentioned here that the capacity method has been criticised on the ground that the dielectric coefficient alters for very small thicknesses, but the recent work of Kollmann and Dorch (*Z. physikal. Chem.*, 1927, **126**, 305) who have been unable to detect any variation for several materials down to 1 μ, renders this unlikely, especially when taken in conjunction with the above results obtained by two independent methods.

Table III gives a few of the results obtained. The value for ϵ is the ratio of the capacities after and before filling with oil allowing for stray capacities. The thickness of the oil film is calculated on the assumption that $\epsilon = 2{\cdot}21$, the value obtained by measurement in a larger apparatus. The figures represent mean thicknesses, but the minimum

thickness is nearly the same (within 0·5 μ) as the cylinder and plate were almost flat. Although the absolute values are subject to an error of this magnitude, the relative values are much more accurate.

TABLE III.

Thickness of Air and Oil Films.

Experiment No.	Fibre, μ	Air film, μ	Oil film, μ	ϵ	Remarks.
1	33 app.	30	30	2·21	Quartz fibre, R very high.
2	2·8	4·5	4·73	2·08	Initial thickness 8·2 μ, value 4·5 after tapping. Oil film 4·15 μ with 50 gm. weight on cylinder. Conduction irregular.
3	2·8	6·36	6·36	2·21	Initial thickness 15·0 μ, 6·36 after prolonged tapping. Conduction partial.
4	12	16·8	16·9	2·20	Oil film reduced to 13·7 μ with 50 gm. weight. No conduction 120 v.
5	9	—	9·9	—	Conducted partially.
6	3	11·0	10·9	2·22	Fibres slightly oily; conducted 6 v.
7	3	9·9	10·1	2·17	Thickness before tapping 13·8 μ. Conducted 6 v.
8	9	11·8	11·6	2·24	Partially conducted 24 v.
9	9	10·1	8·3	2·68	Same fibres, one very near edge possibly washed out by oil. Partly conducted 14 v.
10	2·5	4·95	4·9	2·22	Broke down at 66 v.
11	0	2·11	2·10	2·22	No fibres. Initial ϵ = 2·04. Partly conducted 2 v.

Experiments 2 and 3 clearly show the metastable floating effect, the thickness of the air film having been reduced to approximately one-half by tapping. Experiment 11 was the only one of many attempts to float the cylinder on the plate without fibres which was successful. It was accomplished by washing the surfaces with anhydrous methyl alcohol, wiping with a clean cloth and immediately placing the two together. Under these conditions it is highly probable that the surfaces were contaminated by an adsorbed layer of alcohol. The cylinder sank slowly (0·04 μ in 10 minutes) just as if it were floating on oil, and was fairly stable to tapping. On introducing oil it rose

nearly 0·2 μ, and then sank, at first rapidly, then more slowly, to the original distance, at which point oscillation stopped owing to the high conductivity.

The actual thickness of the air films (4·0 μ) measured by Hardy and Nottage on a contaminated surface is of the same order as those recorded in Table III for very thin fibres. As it has not been possible to obtain a non-conducting layer even with a moderately clean surface, the observation that the film is thicker when the surfaces are clean could not be confirmed. In this connection it may be mentioned that through the courtesy of these authors, we were enabled, while their experiments were in progress, to examine the conductivity of one of their steel plates and cylinders after careful cleaning according to their standard method. The non-conducting state could not be obtained even with the application of only 1·4 volts, but on rubbing the plate with the finger and replacing the cylinder, a very stable non-conducting film, with a resistance of over 10 megohms, was formed and the cylinder could be shaken or moved about on the plate without producing any signs of conduction.

On p. 224 of the paper under reference it is stated: 'If more accurate methods should confirm the figures, the distance between the cylinder and plate would be the same for a gas as for a liquid and independent of the chemical constitution of the lubricant and of the enclosing solids.' If this is true, the constancy should hold good not only for any gas but for any gas at any pressure. The present experiments, although limited in scope, indicate that the agreement under suitable conditions may be as close as 1 per cent. for paraffin oil and air at 685 mm. pressure, and, consequently, it was considered of interest to examine the effect of reducing the pressure. The cylinder was floated on an almost invisible 'wiped' film of the oil and the whole covered with a bell jar which could be evacuated. The film thickness was 4·0 μ. On reducing the pressure to about 100 mm. a slow steady fall set in, but the distance moved was only 0·01 μ in 9 minutes. On readmitting air the cylinder rose 0·005 μ in 7 minutes and then remained fairly steady. The cylinder was then slightly repolished and floated on a fresh contamination giving a film 3·74 μ in thickness. A similar reduction of pressure caused a fall of 0·027 μ in 3 minutes, the subsequent rise to a steady value being 0·022 μ in 3 minutes. Paraffin oil was then run under the cylinder causing the resistance to fall below 300 ohms, but on vigorous tapping it increased to 50,000 ohms, and then to several megohms, and the film thickness was found to be 3·54 μ, a value sufficiently close to the one with air, considering the somewhat rough treatment the cylinder had received.

In another experiment with a larger gap of 12·5 μ, a fall of 0·67 μ, of which half was in the first half minute, was recorded, followed hy. a

rise of 0·38 μ on readmitting air. In this case the cylinder was not fully stabilised by tapping. After three more evacuations to 2 mm. the fall was only 0·07 μ and the rise 0·035 μ. A change in capacity, probably due to the removal of air from the gap and its readmission, could be heard as it was quicker than the movement of the cylinder, but a similar effect would be produced if the cylinder were slightly raised by a release of the pressure on top of it, assuming that the air only issues slowly from the gap. It is not possible to distinguish between the two effects.

These experiments indicate that there is a critical distance between two surfaces at which unexpected phenomena occur. In the case of the cylinder this distance is about 4 μ and for the slider with 3 mm. legs something over 1 μ. The total film thickness depends on the amount of contaminant, as it decreases with wiping or with reduction in size of the glass fibre separators, which appear to act in a similar manner to the oil film, and it might perhaps be expressed as 'effective contaminant thickness + a constant.' The effect is the same as if each surface were covered with a rigid layer and the contamination forced the two apart, but the formation of these layers appears to be facilitated by the presence of the contamination, since it is very difficult to arrive at the non-conducting state with two clean surfaces. If the quantity of contaminant is excessive, as in the case shown in fig. 2, where the larger droplets are about 50 μ in diameter, when the upper body is placed on them, contact with the oil takes place long before the critical distance is reached, and the effect is the same as when the body is placed in a pool of oil, *i.e.*, conduction occurs if the pressure is sufficient. Non-conducting wiped films can only be obtained when the film is practically invisible.

The rigidity of the composite film is evident from the results in Table II and its stability is so remarkable that it is, on first experiencing it, almost impossible to remove the impression that it is due to dirt between the surfaces, but the reason for the formation of the film is obscure. An electrical repulsion, due to the polarisation of the molecules, appears to be excluded, since the distance remains unchanged when a liquid of different dielectric constant is introduced into the gap, and at present no further explanation is forthcoming. A method has been devised for measuring the thickness of conducting films, and it is intended to examine outgassed surfaces in the hope that further light may be thrown on the matter.

To return to the figures in Table *III*: with the exception of experiment 10, which appears to be abnormal, all the films of 11 μ and under in thickness conducted on the application of comparatively low E.M.Fs., but there is no obvious relation between the film thickness

and the breakdown E.M.F. This is perhaps to be expected from the results of the wedge experiments. In nearly all cases the first stage of breakdown was to a partially conducting state in which the resistance varied from about 10,000 ohms to a few megohms. The conduction was frequently of an intermittent nature as the galvanometer needle tended to fluctuate some 20 per cent. from the mean, but, occasionally, particularly with heavier currents, it was steady. There appeared to be no polarisation of the electrodes as no current was obtained on shorting the source of E.M.F., whereas a film of octyl alcohol under similar conditions gave an appreciable reverse current for a few minutes changing in direction with a change in the polarity of the initial E.M.F. The resistance of the film invariably decreased with increase of current, and in several cases there appeared to be a tendency for the potential drop across the film, *e*, to remain constant, some of the results being shown in Table IV where *c* is in microamps and *e* in volts.

TABLE IV.

Conductivity of Partially Conducting Oil Films.

A		B		C		D		E		F		G	
c	*e*	*c*	*e*	*c*	*e*	*c*	*e*	*c*	*e*	*c*	*e*	*c*	*e*
1·5	0·44	0·2	1·8	2·1	6	0·5	9	10	9	16	8	75	13
3·2	0·66	1·1	2·9	2·9	7	4·4	10	69	14	46	19	225	18
4·9	1·0	2·5	3·4	9	11	10	10	125	17	108	19	420	18
7·0	1·0	4·2	3·8	16	14	20	10	220	18	208	19	640	17
		6·2	3·8	22	17					475	18	880	12
												1,100	12

Columns A and B refer to the film of experiment No. 3 of Table III with 1 megohm in series. After making the measurements "A," the plate was tapped and the resistance increased to over 100 megohms when tested by applying 4 volts. At 6 volts there was partial breakdown and readings "B" were taken by applying 2, 4, 6, 8 and 10 volts in succession. On tapping, the resistance again became very high, but the film broke down partially at 4 volts, and with 6 the resistance became very low indeed. This behaviour was typical of the thinner films. The remaining figures all refer to one film, No. 8, of Table III, 11·6 μ in thickness. This film initially broke down with 24 volts. In

a preliminary series of readings the resistance fell from 28 to 9 megohms. On repetition it was found to be only 3 megohms and the readings "C" were obtained. After standing 24 hours the film became non-conducting with a resistance of over 1,000 megohms, but broke down with 62 volts, giving irregular readings. After tapping and allowing to stand for some hours, the fairly steady values "D" were found at a room temperature of 31°. After standing overnight, 14 volts were required to break down the insulation which had again become high and the values "E," "F" and "G" were obtained in succession at 30°.

With both films the E.M.F. increases with the current for small values of the latter, but Ohm's law is not obeyed; with larger currents the E.M.F. becomes nearly constant and on still further increase it decreases. The behaviour in the later stages resembles the discharge in a gas without a positive column in which the E.M.F. remains constant until the cathode is covered with the glow, followed by arcing under suitable conditions. The steady potential appears to depend upon the thickness of the film, but it may also vary considerably for the same film. Until further experiments have been completed, it is not possible to say whether the conductivity is due to impurities in the liquid, to the type of discharge usually associated with dielectric break-down or to some other effect.

Disc Experiments.

As mentioned above, it was found that discs of brass and steel would float on a layer of oil or of air. The latter effect appeared to be produced very readily if the discs were lightly polished with rouge and water on a pitch polisher and then cleaned with anhydrous methyl alcohol. After immersing in oil and cleaning several times, flotation was more difficult to secure. It seems possible that a trace of contamination from the polisher assists the formation of the film, although it was not sufficient to produce an insulating layer in the case of the slider and the cylinder.

The loads per square centimetre with these discs were 3·6 and 3·3 gm. Ormandy (*Proc. Inst. Mech. Eng.*, 1927, **1**, 307) has given some results for a larger disc in a pool of paraffin oil producing a load of 4·7 gm. per square centimetre. His thicknesses have been miscalculated and should be only one-fourth of those tabulated. The films became conducting after some minutes and a minimum thickness of 2 wave-lengths or 1·2 μ was measured, much less than that obtained in the present experiments. In the latter a brass disc placed in a pool of paraffin oil sank in 6 minutes at a temperature of 25° to a distance of 19 μ from the plate, but was still falling slowly. After pressing it sank to 17 μ and remained quite steady. By repeated pressing this distance

was reduced to 12·7 μ, on releasing the pressure (about 500 g.) the disc rose immediately a distance of 0·03 to 0·05 μ.

The same disc floated at a distance of 9 μ from a contamination of myristic acid, while a steel disc initially rested at a distance of 12 μ from a clean plate, sinking very slowly. On tapping it fell to about 4 μ and remained steady. On admitting oil to these air systems in a stable state, values of 2·30, 2·24, 2·24 and 2·33 were obtained for the dielectric constant, showing that the disc was pulled down slightly. On one occasion oil was admitted before tapping and the value found was 2·70, showing a much larger movement. Values for the conductivity analogous to these with the cylinder were obtained, but it was difficult to polish the discs sufficiently flat and, consequently, the figures for the thickness are not very reliable. Further experiments will be made after repolishing.

In conclusion, attention may be drawn to a recent paper by Joffé, (*Trans. Faraday Soc.*, 1928, **24**, 65) in which he states that exceedingly thin films were found to have a very high dielectric strength. In the absence of experimental details it is difficult to compare these results with those recorded in the present communication.

SUMMARY.

Methods are described for determining non-conducting film thicknesses by means of capacity measurements; with suitable arrangements very minute changes in thickness can be measured.

There is a strong tendency for a film of air of the order 1 to 4 μ in thickness to form between two plane surfaces, particularly if one of them is slightly greasy. Hardy's measurements have been confirmed and his hypothesis that the film thickness is unchanged when the air is replaced by oil has been shown to hold good, with certain limitations, to an accuracy of at least 5 per cent. The thickness has also been shown to be nearly independent of the air pressure down to 2 mm.

The coefficient of friction for the air film between contaminated surfaces has been shown to decrease with increase of pressure, although the distance between the surfaces remains nearly constant and there is no relation between the coefficient of friction and the thickness.

Air films have a high insulation resistance and when they break down the resistance usually becomes very low. Flooded films of paraffin oil will stand a high potential gradient (over 100,000 v./cm.) as long as their thickness is more than about 15 μ. At 11 μ there is a

tendency to break down with a much smaller potential gradient and the breakdown thickness does not vary greatly with the voltage. The film becomes conducting in two stages; in the first, the resistance is of the order of 1 megohm, Ohm's law is not obeyed, but there is a tendency for the E.M.F. across the film to remain constant with variation of currents over a certain range. In the second stage the resistance is very low.

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THE PHOTO-VOLTAIC PROPERTIES OF PHOTOTROPIC MERCURY COMPOUNDS.

By Bh. S. V. Raghava Rao.

In previous publications (*J. Indian Inst. Sci.*, 1929, **12A**, 1, 17) a description has been given of a number of phototropic mercury compounds and their photoelectric properties. No very definite relation appears to exist between the time a substance takes to darken and its photoelectric emission, a result which may be due to the fact that the darkening is effected by visible light, whereas photoelectric emission only occurs on exposure to ultra-violet rays. It seemed possible, however, that there might be some correlation between the analogous phenomenon of photo-voltaic potential and one of the above properties; and consequently it was decided to make potential measurements with this object in view.

It was found in most cases that two potentials had to be dealt with, the first being that between the two phototropic forms in the dark and the second the one produced on illuminating one electrode while the other was kept in the dark. Owing to the dark potential between the two forms it was not possible to determine the photo-potential of the pale form of the substance and measurements had to be confined to the dark forms resulting from exposure of the freshly prepared substances to light. Although differences in potential reaching 0·1 volt have been measured, no relation has been found between these and other properties of the compounds investigated.

EXPERIMENTAL.

The photo-voltaic cell consisted of two electrodes of the sensitive substance immersed in an electrolyte in the two arms of an H-tube. These electrodes were made of platinum foil welded to a platinum wire sealed into the end of a narrow glass tube filled with mercury for making contact with the leads. The back and edges of the foil as well as the connecting wire were coated with a thin layer of paraffin to prevent local cell formation. The sensitive substance was made into a thick paste with alcohol-ether mixture to which a few drops of collodion had previously been added, coated on the platinum foil and dried in the dark. The electrolyte, finally adopted after a series of trials with several neutral salts and acids (alkalis being out of consideration as they were known to decompose the substances), was an $N/100$ solution of acetic acid previously saturated with the sensitive substance. The cell was covered with black paper leaving a small opening in one of the H-arms for illuminating the electrode.

The source of light was a 100 c. p. pointolite lamp, the light from which traversed a layer of distilled water 5 cms. thick in a neutral glass cell before reaching the electrode. The distance between the light source and the illuminated electrode was ordinarily 12 cms., ranging from 10 to 14 cms. in the case of measurements on temperature coefficients.

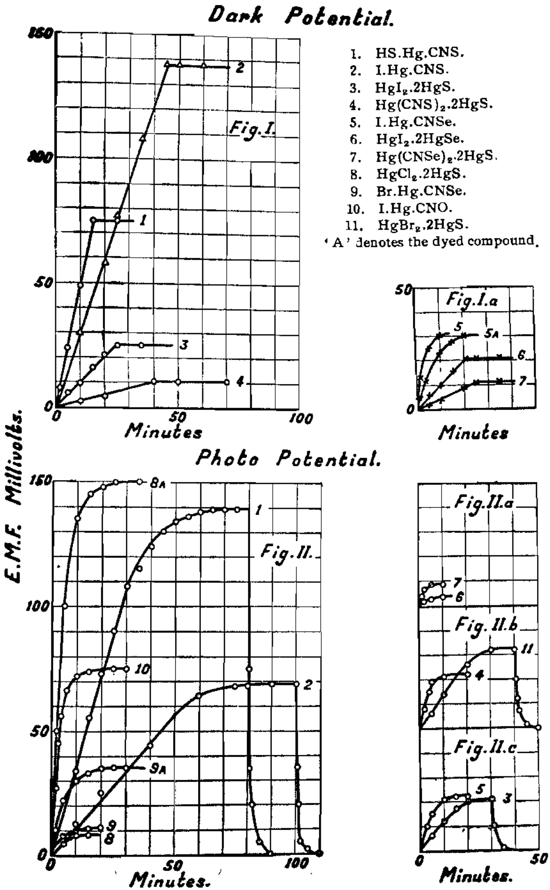
Potential differences were measured with a Tinsley vernier potentiometer and mirror galvanometer which effected measurements of potential to 1 micro-volt. This degree of accuracy was not essential since the E. M. Fs. obtained were relatively high and the limits of reproducibility with different cells employing the same compound ranged from 5 to 10 per cent, but the sensitiveness was of value while measuring the change of E. M. F. with time.

THE 'DARK' E. M. F. BETWEEN TWO PHOTOTROPIC FORMS.

Of eleven compounds studied, seven were found to give rise to an E. M. F. when their two phototropic forms were made the electrodes in a cell not exposed to light. In all cases the light-coloured phototrope was positive.

By placing two freshly prepared electrodes in a cell and exposing one of them to light for various times, it was possible to measure the E. M. F. between the unaffected compound and its mixtures with varying proportions of the darkened variety. After illuminating the electrode it was necessary to wait a short time for the photo-potential to die down. The results are shown diagrammatically in Fig. I, and it will be noticed that in all cases except that of the compound I.Hg.CNSe, the E. M. F. initially bore a linear relation to the time of exposure, that is, to the quantity of dark compound formed. After some time a saturation point was reached. This occurred when a comparatively small proportion of the substance had been converted into its phototrope. For the compounds I.Hg.CNS, HS.Hg.CNS and HgI_2. 2HgS the conversion was estimated by the colour to be about 20 per cent., and from the relative rates of darkening which are approximately known, the amount of dark-coloured Hg $(CNS)_2$. 2HgS formed at the maximum E. M. F. was somewhat less than this and in the case of the selenium compounds I.Hg.CNSe, HgI_2. 2HgSe and $Hg(CNSe)_2$. 2HgS was only about 6 per cent.

These results are analogous to those of Winther (*Z. Electrochem.*, 1912, **18**, 138) for the photolytic oxidation of Fe(ous) into Fe (ic) in presence of mercuric chloride; and of Bourgeand (*Compt. rend.*, 1926, **182**, 1619) for the transformation of black mercuric sulphide into the red modification.



Dark Potential.
Fig. I.
Fig. I. a
1. HS.Hg.CNS.
2. I.Hg.CNS.
3. HgI₂.2HgS.
4. Hg(CNS)₂.2HgS.
5. I.Hg.CNSe.
6. HgI₂.2HgSe.
7. Hg(CNSe)₂.2HgS.
8. HgCl₂.2HgS.
9. Br.Hg.CNSe.
10. I.Hg.CNO.
11. HgBr₂.2HgS.
'A' denotes the dyed compound.
E.M.F. Millivolts.
Minutes
Photo Potential.
Fig. II.
Fig. II. a
Fig. II. b
Fig. II. c
Minutes.

THE PHOTO-POTENTIAL.

When the two electrodes are of the same blackened form, the potential difference between them is zero, or very nearly so. On illuminating one, the potential rises rapidly at first and then more slowly until a limiting value is reached. The direction of the photo-current inside the cell is from the dark electrode to the illuminated one.

Fig. II shows some typical curves for the rise of photo-E. M. F. during illumination. The time taken to reach the maximum potential varies with the compound and bears no relation to the phototropic activity or to the magnitude of the photo-E. M. F. The results are given in Table I.

TABLE I.

Phototropic Activity and Photo-potential.

Compound	Time of darkening in light, mins.	Time to reach steady potential, mins.	Maximum photo-potential, milli-volts
HS.Hg.CNS ...	4	50	139
I.Hg.CNO ...	11	10	75
I.Hg.CNS ...	8	60	69
$HgBr_2$. 2HgS ...	11	25	33
I. Hg. CNSe ...	9·5	10	22
Hg $(CNS)_2$. 2HgS ...	10	5	22
HgI_2. 2HgS ...	5	20	21
Br.Hg.CNSe ...	12	15	11
Hg $(CNSe)_2$. 2HgS ...	16	4	9
$HgCl_2$. 2HgS ...	40	8	8
HgI_2. 2HgSe ...	13	5	4

On removing the light, the potential of the illuminated electrode falls to about half its maximum value in the first 30 seconds and then decreases more slowly. The initial condition (that before illumination) is reached in every case in less than 10 minutes. Fig. II also shows some typical curves for the fall of potential.

RELATION BETWEEN THE PHOTO-POTENTIAL AND THE WAVELENGTH OF THE EXCITING LIGHT.

By placing filters in the path of the light falling on the illuminated electrode it was possible to form an estimate of the action of light of different wavelengths. Five Wratten filters of known transmission characteristics were used. Two of these, Nos. 47 and 61, transmitted a comparatively narrow band, the limits for 50 per cent. of the maximum

transmission being 4,000-4,850 and 5,050-5,600 A.U., respectively. The other three, Nos. 3, 8 and 23 *A* produced a sharp cut-off at 4,600, 4,880 and 5,780 A.U., but were transparent for longer wavelengths. By taking the difference of the E. M. Fs. with two of these filters, an approximate value for the intervening band could be obtained. In order to make the results for different filters comparable, they had to be corrected for the total transmission. This could be deduced from the known absorption curves of the filters, relative values for Nos. 47 and 61 being 0·42 and 0·29, white light on the same scale between the limits 3,800 and 6,500 A.U. having the value 2·7; 6,500 was chosen as the limit on the long wave side, as there appeared to be very little action produced by light of greater wavelength.

Table II gives the relative values in millivolts calculated by dividing the E. M. Fs. by the total transmission, and indicates the wavelength of the centre of the transmission band.

TABLE II.

Photo-potential for different wavelengths.

Filter		47 ÷ 0·42	(3-8) ÷ 0·27	61 ÷ 0·29	(8-23A) ÷ 0·68	23A ÷ 0·61	÷ 2·7
Wavelength		4,425	4,750	5,325	5,400	5,750	white
HS.Hg.CNS	No dye	116	68	12	6·5	..	52
	Dyed	95	197	24	19	10·5	50
$HgI_2.2HgS$	No dye	>47	...	>69	...	...	>7·5
I.Hg.CNS	No dye	72	46	48	27	7·5	25
I.Hg.CNSe	No dye	...	11	...	13	8	7·5
Br. Hg.CNSe	No dye	7	7·5	...	1·5	2·5	3
	Dyed	24	19	...	28	10	13
$Hg(CNS)_2.2HgS$	No dye	24	7·5	5	...	...	8
	Dyed	27	9	14	3	5·5	9
:. Hg.CNO	No dye	99	...	62	...	23	28
$HgBr_2·2HgS$	No dye	21	...	...	...	...	12
	Dyed	35	19	65	32	16	17
$HgCl_2.2HgS$	No dye	9·5	6·5	0	0·3	0	3
	Dyed	>342	...	...	...	23	>53

In several cases the compounds were dyed with erythrosin, as such treatment had been found to exert a marked effect on the light-sensitiveness. In the table, the order in which the substances are arranged is according to their sensitiveness to light when undyed, the most sensitive being placed first. Measurements were made of the times required to darken to a standard shade through the various screens, but as the results are of barely more than qualitative value, they have not been included, more definite information having already been obtained spectroscopically (*J. Indian Inst. Sci.*, 1929, **12A**, 1).

The undyed compounds exhibit a certain regularity, the maximum effect occurring with the shorter waves. This is analgous to the phototropic activity, but the maximum appears to lie nearer the ultra-violet in the case of the photo-potential. The results for the dyed compounds are more irregular, but there is a shift of the maximum towards the red. It is possible that two maxima are present as there are for the phototropic activity and that these cause the apparent irregularities. The effect of dyeing is very marked in most cases and particularly for the compound $HgCl_2.2HgS$, the most insensitive towards light as regards colour change. The E. M. F. was 145 mv. with all screens except No. 23 A, so that saturation was reached (see next section) and the relative values could not be calculated. The case of $HgI_2.2HgS$ was similar, although the saturation value was much smaller, only 20 mv.

The actual values for the photo-potential from different compounds appear to be entirely independent of the nature of the compound and of its photoelectric or phototropic sensitiveness.

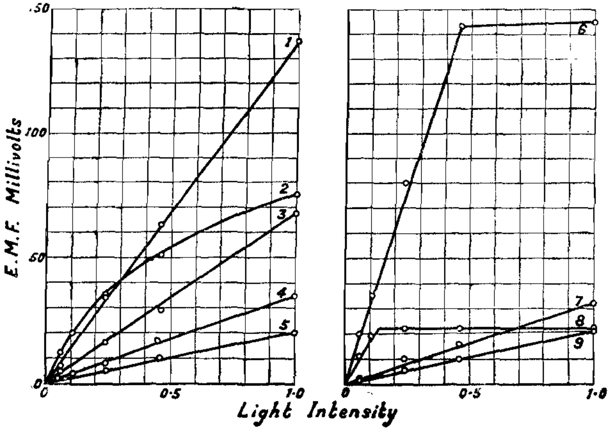
Relation between Photo-potential and Light Intensity.

Measurements were made of the photo-potential variation with the intensity of the light, the amount of light reaching the electrode being varied by interposing fogged photographic plates of different degrees of transmission. It has been shown by Edison Pettit (*Astrophys. J.*, 1927, **66**, 43) that such plates have nearly the same transmission for all wavelengths in the visible so that the light does not change appreciably in colour after passing through them. The transmission powers of the plates were measured by comparison with a neutral tint wedge. Fig. III shows the relation between intensity and light potential. Curves 1, 3, 4, 5, 7, 9 are for the compounds HS·Hg.CNS, I.Hg.CNS, Br.Hg.CNSe (dyed), I.Hg.CNSe, $HgBr_2.2HgS$ and $Hg(CNS)_2.2HgS$. Curves 6 and 8 for the compounds $HgCl_2.2HgS$ (dyed) and $HgI_2.2HgS$ while showing at low

intensities a steady rise of E. M. F. with intensity of light exhibit *saturation points. A different type of curve is given by I.Hg.CNO (2) the reason not being apparent.*

It appeared likely that other substances showing no saturation might exhibit the phenomenon at higher intensities and this was confirmed in the case of compound HS.Hg.CNS by exposing it to the light of a carbon arc. A saturation E. M. F. of 170 mv. was reached with an intensity 20 per cent. greater than that of the pointolite lamp.

Fig. III.
E.M.F. and Light Intensity



1. HS.Hg.CNS.
2. I.Hg.CNO.
3. I.Hg.CNS.
4. Br.Hg.CNSe(dyed)
5. I.Hg.CNSe.
6. $HgCl_2$.2HgS (dyed).
7. $HgBr_2$.2HgS.
8. HgI_2.2HgS.
9. $Hg(CNS)_2$.2HgS.

INFLUENCE OF TEMPERATURE ON THE DARK AND PHOTO-POTENTIALS.

A series of measurements was made with the object of determining the temperature variation of the dark and photo-potentials. For this purpose the cell was placed inside a small electrically heated oven with a glass front. Measurements of the potentials were taken at 30°, 40° and 50°. The results are given in Tables III and IV.

TABLE III.

Temperature and Dark Potential.

	Potential difference between the unexposed and the previously exposed electrode, millivolts at		
	30°	40°	50°
I. Hg. CNS ...	142	141	138
HS. Hg. CNS ...	74	75	74
I. Hg. CNSe ...	32	31	32
HgI_2. 2HgS ...	24	24	25
Hg $(CNS)_2$. 2HgS ...	23·5	22	20

TABLE IV.

Temperature and Photo-potential.

—	Photo-potential, millivolts				
—	30°	40°	50°	E_{40}/E_{30}	E_{50}/E_{40}
$HgCl_2$. 2HgS (dyed) ...	154	115	85	0·75	0·75
HS Hg. CNS ..	131	133	133	1·0	1·0
I. Hg. CNS ...	62	60	59	1·0	1·0
I. Hg. CNO ...	56	41	32	0·75	0·8
Hg Br_2. CNSe (dyed) ...	34	34	32	1·0	1·0
$HgBr_2$. 2HgS ..	30	19·5	12·5	0·65	0·65
HgI_2. 2HgS ...	20	17·5	15·5	0·9	0·9
Hg $(CNS)_2$. 2HgS ...	18	16·5	15·5	0·9	0 95
I. Hg. CNSe ...	14	12·6	11·5	0·9	0·9
Hg $(CNSe)_2$. 2HgS ...	6·5	5·2	1·4	0·8	0·85

It would appear that the 'dark potential' for any of the compounds is not appreciably influenced by temperature within the range 30–50°. The photo-potential is also in the majority of cases independent of temperature, while in some instances, remarkably with $HgBr_2$. $2HgS$, it has a negative temperature coefficient. Compounds exhibiting this behaviour are relatively insensitive as regards

colour changes and it is probable that at the higher temperatures the reverse change of colour is sufficiently rapid for a considerable proportion of the incident light energy to be spent in blackening the compound with the result that the photo-potential is lower.

SUMMARY.

1. The behaviour of eleven phototropic mercury compounds in a photo-voltaic cell has been examined. Of these seven have been found to exhibit a 'dark potential' between the light and dark-coloured forms.

2. The 'dark potential' is in general proportional to the time of previous exposure of the electrode substance, that is to say, to the quantity of dark-coloured compound, up to a definite limit above which it remains constant.

3. On exposure to light the photo-potential gradually increases to a maximum and remains steady without any sign of fatigue. On cutting off the light, the original condition is reached rapidly, the fall of potential being about 50 per cent. in the first 30 seconds.

4. The photo-potential is in many cases proportional to the intensity of light and shows a saturation point above which a further increase in the intensity of light does not change the potential. The maximum photo-potential measured was 0·170 volt in the case of the compound HS.Hg.CNS.

5. In the visible region of the spectrum, light of the shortest wavelengths produces the maximum photo-potential; dyeing in erythrosin gives rise in nearly all cases to an increased value of the potential and the maximum effect is shifted towards the red.

6. The temperature coefficient of the 'dark potential' is found to be nearly 1 for five compounds in the temperature range 30–50°. The photo-potential for three compounds has been found to be independent of temperature, while for seven, a rise of temperature lowered the potential.

My best thanks are due to Professor H. E. Watson for continued help and guidance during the progress of the work. I also take this opportunity to thank the Andhra University for the award of a fellowship, which enabled me to conduct this research.

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I.—CONTRIBUTIONS TO THE STUDY OF SPIKE-DISEASE OF SANDAL (*SANTALUM ALBUM*, LINN.). PART VII.

Factors influencing Diastatic Activity.

By B. N. Sastri and M. Sreenivasaya.

It was pointed out (*J. Indian Inst. Sci.*, 1928, **11A**, 23) that the higher diastatic activity of the spiked leaves of sandal was not due only to the higher concentration of diastatic enzymes, but was possibly enhanced by (1) the low P_H value approximating to the optimum reaction of the plant amylases, (2) the lower calcium content and (3) the higher amino-nitrogen content.

It is generally known that phosphates, nitrates, chlorides and amino-acids accelerate the diastatic activity (Lintner, *J. pr. Chem.*, 1887, **36**, 481 ; Effront, *Compt. rend.*, 1892, **115**, 1324 ; Cole, *J. Physiol. Chem.*, 1904, **30**, 202–281), while calcium is supposed to exert an inhibiting influence. An attempt has been made to study (1) the relative concentration of the diastatic enzymes in the healthy and diseased leaves, (2) the enhancement of activity due to various activators and (3) the possible presence of inhibitors in healthy leaves.

PREPARATION OF THE ENZYME EXTRACT.

Healthy and diseased leaves were obtained from the two areas, Ragihalli and Uttarahalli, referred to in our previous communication, desiccated over calcium chloride, powdered to pass through a 40-mesh sieve and preserved in stoppered bottles. The enzyme extracts were prepared in the same way as already described (*J. Indian Inst. Sci.*, 1928, **11A**, 27).

DIALYSIS AND DETERMINATION OF RELATIVE CONCENTRATION OF DIASTATIC ENZYMES.

Definite volumes of the extracts were dialysed in collodion bags against distilled water at room-temperature for 48 hours. The activity of the dialysed enzyme extract, freed from a large proportion of the crystalloid activators, was measured in the usual way, and the relative concentration of the diastase in the two extracts tabulated, assuming that the amount of maltose produced in equal intervals of time is proportional to the concentration of the enzymes at initial stages of action.

TABLE I.

Activity of Dialysed Enzyme Extracts.
(Ragihalli, November 27, 1929).

Reaction mixture, 50 c.c. each of enzyme and 4 per cent. soluble starch: temperature, 30°. U = Undialysed. D = Dialysed.

Time in minutes	Mgms. of maltose per 20 c.c. of reaction mixture							
	Healthy		Spiked		Relative concentration of spiked to healthy		Percentage loss of activity by D	
	U	D	U	D	Before D	After D	Healthy	Spiked
30	6·3	4·4	16·5	6·3	2·6	1·4	30·1	61·8
60	8·1	4·8	18·0	7·6	2·2	1·5	40·7	57·7

It is clear from the table that (1) loss in activity is higher in spiked than in healthy extract and (2) the diastase content of the spiked leaf extract is 1·5 times that of the healthy leaf extract.

STUDY OF ACTIVATORS.

Table I shows that the loss of activity in the diseased leaf extract is about 60 per cent., while the corresponding figure for healthy leaf extract is about 35. This is largely due to the escape of diffusible activators during dialysis and a change in the hydrogen-ion concentration. The results of analysing extracts are incorporated in Table II.

TABLE II.

Analysis of the Enzyme Extracts (per 100 c.c.).

	Ragihalli 27—11—27		Uttarahalli 9—11—28	
	Healthy	Spiked	Healthy	Spiked
Total solids ...	3·81 g.	4·80 g.	4·54 g.	4·42 g.
Ash ...	740 mg.	450 mg.	880 mg.	440 mg.
Phosphate (P_2O_5) ...	20·8 ,,	37·7 ,,	47·0 ,,	62·0 ,,
Calcium (Ca) ...	...	...	139·0 ,,	17·5 ,,
Total Nitrogen ...	60·3 mg.	101·5 mg.	235·8 ,,	425·0 ,,
Amino-nitrogen ...	22·4 ,,	42·3 ,,	34·7 ,,	43·2 ,,

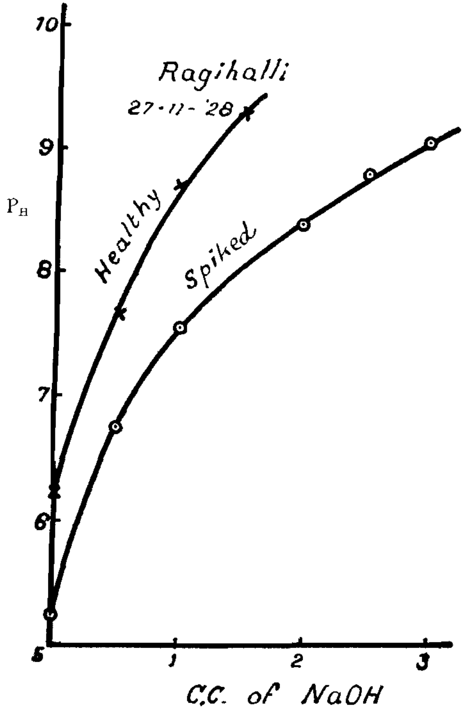


FIG. 1.

A lower ash content, a higher proportion of phosphorus, a higher total and amino-nitrogen, and a lower calcium content characterises the enzyme extract from the spiked leaves. The nitrate nitrogen is practically absent from the spiked leaf extract, but is present in the healthy extracts. The chloride content is the same in both cases.

The higher proportion of phosphorus and the higher amino-nitrogen content of the spiked leaf extract accelerate the diastatic activity, while the low calcium content is also favourable.

INFLUENCE OF P_H ON THE DIASTATIC ENZYMES.

The hydrogen-ion concentration and the buffer value of the original extracts are tabulated below.

TABLE III.

Buffering Capacity of Leaf Extracts.

(Ragihalli, November 27, 1927).

Electrometric titration of extract (5 c.c.) with 0·1 *N* NaOH.

NaOH, c.c. ...	0·0	0·5	1·0	1·5	2·0	2·5	3·0
Healthy P_H ...	6·24	7·65	8·70	9·39	...	...	...
Spiked P_H ...	5·25	6·75	5·75	...	8·36	8·77	9·05

The results are represented in Fig. I. Spiked leaf extract has a lower P_H and a higher buffer value, as can be observed from the curves.

TABLE IV.

Change of P_H on Dilution.

(Ragihalli, November 27, 1927).

Extract	Original P_H	Dilution with equal volume of water	Dilution with equal volume of 4 per cent. starch
Healthy ...	6·24	6·00	5·90
Spiked ...	5·25	5·30	5·30

Table IV clearly shows that the addition of an equal volume of starch does not alter the P_H in the case of spiked extract, while in the healthy extract the fall is 0·3 unit.

Acetate buffers were used to equalise the P_H values of the two extracts during starch hydrolysis. Mixtures employed for the experiment and the results obtained have been tabulated below and also graphically represented (*Fig. II*).

TABLE V A.

Influence of P_H *on Diastatic Action.*

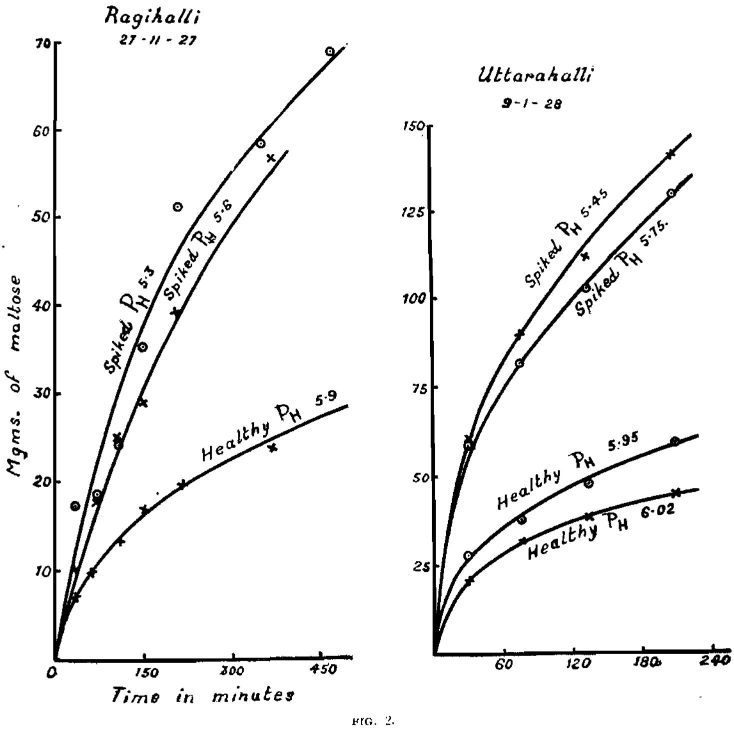
(Ragihalli, November 27, 1927). Reaction mixture, 50 c.c. enzyme, 20 c.c. water or buffer and 30 c.c. of 6·66 per cent. starch solution: temperature, 30°.

Time in minutes	Mgms. of maltose per 20 c.c. of reaction mixture			
	Healthy		Spiked	
	P_H 5·9	P_H 5·85	P_H 5·3	P_H 5·6
30	6·3	...	16·5	...
35	...	6·7	...	10·2
60	8·1	9·7	18·0	17·2
105	11·0	13·0	24·8	25·0
150	18·7	17·0	36·0	28·7
210	20·5	20·8	50·8	39·5
345	23·4	...	58·5	...
360	...	24·4	...	57·0
450	29·7	...	69·1	...

TABLE V B.

(Uttarahalli, January 9, 1928).

—	P_H 6·02	P_H 5·95	P_H 5·45	P_H 5·75
30	21·2	27·0	61·0	59·7
75	31·5	38·2	90·5	82·5
135	38·5	48·6	113·8	103·7
210	45·0	59·6	142·5	126·2



Ragikalli
27 - 11 - 27
Mgms. of maltose
Time in minutes
Spiked PH 5·3
Spiked PH 5·6
Healthy PH 5·9
Uttarahalli
9 - 1 - 28
Spiked PH 5·45
Spiked PH 5·75.
Healthy PH 5·95
Healthy PH 6·02

FIG. 2.

It is clear from tables and figures that the hydrogen-ion concentration has influenced the activation, and that the higher buffer action of the spiked leaf extract tends to maintain the optimum P_H and resists the action of appreciable quantities of acetate buffer.

PRESENCE OF INHIBITORS.

Experiments were made to reveal any change in activity by employing mixtures of tissue fluids from the healthy and spiked leaves. The presence of inhibiting agents in healthy leaf extracts would bring about a measurable reduction of activity of the spiked leaf extract on adding boiled extract of healthy leaves. The results have been tabulated below. The results are given in c.c. of $KMnO_4$ (1 c.c. = 10 mg. of copper) required for the estimation of sugars by Bertrand's method produced in 20 c.c. of the reaction mixture after incubation at 30° for 20 hours.

TABLE VI.

Influence of Boiled Extracts on Active Extracts.

(Uttarahalli, August 15, 1927).

Reaction mixture	c.c. of $KMnO_4$	Coloration with Iodine
25 c.c. of 2 per cent. starch, 2 c.c. spike extract and 2 c.c. water	9·30	Red
25 c.c. of starch, 2 c.c. boiled spike extract and 2 c.c. water	1·50	Blue
25 c.c. of starch, 2 c.c. healthy extract and 2 c.c. water	3·30	Reddish violet
25 c.c. of starch, 2 c.c. boiled healthy extract and 2 c.c. water	1·60	Blue
25 c.c. of starch, 2 c.c. boiled healthy extract and 2 c.c. spike extract	10·80	Red
25 c.c. of starch, 2 c.c. boiled healthy extract and 2 c.c. boiled spike	3·05	Blue
25 c.c. of starch, 2 c.c. healthy extract and 2 c.c. boiled spike	6·15	Red

Healthy leaf diastase is activated by the addition of boiled spiked leaf extracts, and addition of boiled healthy leaf extracts to active spiked leaf extract does not inhibit the activity of the latter. The

experiments, while definitely establishing the presence of activators in the spiked leaf extract, do not indicate the presence of inhibitors in the healthy leaf extract.

The diastatic activity is considerably retarded by the presence of oxidases, as has been shown by True, Black, Kelly and others (*J. Agric. Res.*, 1918, **15**, 369). The leaf extracts have been examined for their oxidase content, and it has been found that the spiked leaf extracts have a decidedly higher oxidase activity. The high starch-splitting activity of spiked leaf extracts, in spite of their high oxidase content, is noteworthy.

MALTASE.

The high cupric-reducing power of the reaction mixture containing spiked leaf extracts might be due to the presence of glucose, produced by the action of maltase on the maltose which is a product of starch hydrolysis by diastase. Extracts were tested for maltase by the usual polarimetric method, and although present, it did not exist in any significant quantities in either of the extracts. The healthy extracts showed a slightly higher maltose-splitting activity than the spiked leaf extracts.

SUMMARY.

The higher diastatic activity of spiked tissues, as compared with the corresponding healthy tissues, is due partly to a higher concentration of the enzyme, partly to the presence of activators like phosphates and amino-acids, and partly to their higher acidity approximating to the optimum P_H of plant diastase.

The lower diastatic activity of the healthy tissues as compared with the corresponding spiked tissues is not due to the presence of inhibitors which retard their amyloclastic capacity.

In conclusion, our thanks are due to Prof. Roland V. Norris for his keen interest and many helpful suggestions in the course of the work.

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II.—CONTRIBUTIONS TO THE STUDY OF SPIKE-DISEASE OF SANDAL (*SANTALUM ALBUM*, LINN.). PART VIII.

Chemical composition of tissue fluids from the leaf.

By M. Sreenivasaya and B. N. Sastri.

The tissue fluid of a living plant represents its dynamic or physiologically active component, while the residue is composed of the static reserves incorporated with the inert structural units of the plant. Specific alterations in the composition of animal blood are brought about with the progressive onset of specific diseases and are often utilised to diagnose and determine the stage of the malady. In plants also marked changes may be expected to accompany the pathological state and a means may thus be found to recognise plant diseases by a biochemical analysis of its tissue fluids long before the external symptoms manifest themselves.

In some diseases prominent changes may take place in leaf tissue, while in others there may be profound disturbances in stem or root. The type of the main metabolic disturbance in the plant may vary with the nature of the disease. In some cases it is the carbohydrate metabolism that may be disturbed, while in other instances the nitrogen or mineral nutrition may have to be considered. In devising methods for diagnosis and for following the progress of plant diseases, it is therefore essential to consider (1) the choice of a suitable tissue that is most affected and (2) the type of metabolism that is most disturbed.

While the changes revealed in the tissue are specific, it should not be overlooked that the causal agency may lie in a different tissue, remote from that under examination, or may even be located externally to the plant. The biochemical changes effected in the diseased tissue fluids are therefore secondary in character, and a closer knowledge of these changes is essential for effectively combating the disease. An attempt has been made to analyse the tissue fluids of sandal in health and disease.

MATERIALS AND METHODS.

The material was collected periodically from two areas, Uttarahalli and Ragihalli, and a full description of its collection, storage, transport and treatment in the laboratory appears in our previous contributions (*J. Indian Inst. Sci.*, 1928, **11A**, 23). The expressed tissue

fluid was centrifuged at 3000 r. p. m. and the clear centrifugate used for analysis. Aliquot portions were used for the determination of total solids, ash, total nitrogen and reducing sugars. Phosphorus and calcium were determined in the ash by the official methods of the A. O. A. C.

TABLE I A.

Yield of tissue fluids from leaves (Uttarahalli).

Date	Percentage yield on weight of green material		
	Healthy	Spiked	Difference
27—4—27	54·0	41·3	+ 12·7
16—6—27	67·1	56·6	+ 10·5
5—8—27	63·2	54·5	+ 8·7
20—4—28	49·0	46·7	+ 2·3
30—4—28	60·6	57·5	+ 3·1
8—5—28	62·4	61·6	+ 0·8
22—5—28	62·5	65·4	− 2·9
8—6—28	57·0	62·7	− 5·7
22—6—28	49·8	56·0	− 6·2
9—7—28	57·1	54·9	+ 2·2
Mean ...	58·2	55·7	+ 2·5

TABLE I B.

Yield of tissue fluids from leaves (Ragihalli).

Date	Percentage yield on weight of green material		
	Healthy	Spiked	Difference
8—7—27	63·7	57·1	+ 6·6
22—7—27	50·4	52·2	− 1·8
29—7—27	55·5	47·3	+ 8·2
Mean ...	56·5	52·2	+ 4·3

TABLE II A.

Grams per 100 c.c. of tissue fluid (Uttarahalli).

Date	Healthy			Spiked		
	Total solids	Ash	Per cent. of ash in solids	Total solids	Ash	Per cent. of ash in solids
19—5—27	13·13	2·21	16·78	13·79	2·20	15·99
16—6—27	10·53	1·93	18·35	12·23	1·45	11·83
23—6—27	11·53	2·04	17·66	10·21	1·52	14·84
15—7—27	12·20	1·92	15·75	12·24	1·66	13·53
5—8—27	13·39	2·91	21·72	12·84	1·98	15·41
18—8—27	15·36	3·62	23·54	14·07	2·26	16·03
15—9—27	13·39	2·91	21·72	12·84	1·98	15·41
27-10—27	16·79	3·63	21·64	14·92	2·18	14·57
9—1—28	15·67	3·71	23·64	16·38	2·12	12·93
11—4—28	17·72	4·46	25·66	14·31	2·41	16·84
20—4—28	20·20	4·53	22·42	17·40	3·77	21·66
30—4—28	15·89	3·59	22·57	14·61	3·08	21·06
7—5—2.	17·67	3·73	21·12	13·20	2·94	22·27
22—5—28	16·42	3·83	23·32	13·06	3·42	26·20
8—6—28	21·25	4·58	21·55	13·43	3·38	25·18
22—6—28	17·48	3·79	21·65	15·52	2·99	19·25
Mean ...	...	3·33	...	...	2·46	...
Standard deviation ...	...	±0·28	...	...	±0·22	...

TABLE II B.

Grams per 100 c.c. of tissue fluid (Ragihalli).

Date	Healthy			Spiked		
	Total solids	Ash	Per cent. of ash in solids	Total solids	Ash	Per cent. of ash in solids
8—7—27	10·90	1·76	16·18	14·95	1·52	10·15
22—7—27	16·36	2·28	13·94	19·24	1·42	7·38
29—7—27	19·57	3·06	15·64	22·55	1·80	7·97
11—8—27	16·09	2·11	13·10	20·10	1·96	9·75
6—9—27	19·62	3·46	17·64	19·95	1·48	7·43
14-10—27	18·81	3·54	18·81	21·90	1·55	7·07
7-11—27	20·92	3·90	16·65	18·92	1·57	8·31
15—6—28	16·71	2·72	16·27	17·36	1·70	9·81
Mean ...	...	2·85	...	...	1·62	...
Standard deviation ...	...	±0·26	...	...	±0·21	...

TABLE III A.

Milligrams per 100 c.c. of tissue fluid (Uttarahalli).

Date	Healthy			Spiked		
	Total N	P_2O_5	Ca	Total N	P_2O_5	Ca
19—5—27	395·2	84·1	...	517·6	81·1	...
16—6—27	357·3	52·3	300·5	659·7	89·3	126·5
23—6—27	405·1	88·1	290·0	496·7	120·1	226·5
15—7—27	379·1	104·5	254·7	533·9	137·4	87·3
5—8—27	429·1	...	...	546·4	...	...
18—8—27	450·8	74·6	257·5	453·0	110·5	52·5
15—9—27	342·0	103·5	165·0	362·6	166·0	107·5
27-10—27	308·8	...	...	512·9	...	...
9—1—28	408·4	...	...	585·2	...	...
11—4—28	332·8	177·8	462·0	623·7	191·8	142·0
20—4—28	466 6	158·4	750·0	659·4	364·6	154·0
30—4—28	400·1	128·2	634·0	616·2	236·0	154·0
7—5—28	328·4	197·1	668·0	542·3	256·0	118·0
22—5—28	327·2	102·7	558·0	543·8	231·0	154·0
8—6—28	246·8	84·2	940·2	499·9	94·9	120·7
22—6—28	307·1	85·5	540·4	464·7	96·8	96·8
Mean ...	367·8	110·7	485·0	538·6	167·3	128·3
Standard deviation ...	± 57·2	± 41·1	± 200·1	± 76·2	± 81·4	± 41·6

TABLE III B.

Milligrams per 100 c.c. of tissue fluid (Ragihalli).

Date	Healthy			Spiked		
	Total N	P_2O_5	Ca	Total N	P_2O_5	Ca
8—7—27	215·6	...	146·3	316·1	...	49·0
22—7—27	135·0	...	86·2	389·8	...	68·8
29—7—27	135 5	122·4	121·7	377·5	112·0	66·4
11—8—27	174·2	95·5	90·9	294·0	29·5	51·4
8—9—27	166·6	104·0	...	370·2	96·4	...
14—10—27	140·0	116·3	131·4	344·9	83·3	48·9
7—11—27	178·0	...	...	253·9	...	...
15—5—28	62·7	...	...	238·6	...	...
15—6—28	271·7	106·7	165·9	561·6	88·9	91·3
Mean	164·3	108·9	123·7	349·6	82·0	62·6
Standard deviation	±54·8	±9·4	±28·4	±90·4	±28·0	±15·2

TABLE IV A.

Milligrams per 100 c.c. of tissue fluid (Uttarahalli).

Date	Healthy			Spiked		
	Reducing sugars	Sucrose	Maltose	Reducing sugars	Sucrose	Maltose
23- 6-27	45·0	135·4	291·7	289·8	337 5	191·3
27-10-27	29·5	500·4	125·0	100·5	604·3	287·5
9- 1-28	51·0	315·5	287·0	220·5	537·8	450·0

TABLE IV B.

Milligrams per 100 c.c. of tissue fluid (Ragihalli).

Date	Healthy			Spiked		
	Reducing sugars	Sucrose	Maltose	Reducing sugars	Sucrose	Maltose
8- 7-27	177·7	291·0	478 5	210·0	163·5	528·7
22- 7-27	210 0	305·0	920·0	338·0	180·0	360·0
14-10-27	...	575·0	705·0	117·5	265·0	870·0

Discussion of Results.

Table I gives the percentages of the tissue fluid that can be expressed under definite standardised conditions and calculated on the weight of the green material. In the majority of cases, healthy leaves yield higher percentages of fluid than spiked leaves and the mean value for healthy is higher than that of spiked.

The total solids and ash per 100 c.c. of tissue fluid, with percentage of ash on the weight of the total solids, have been tabulated in Tables II A and B. It has been pointed out that, especially in view of the parasitic nature of sandal, it is misleading to compare the samples brought from one area with those of another. We have accordingly divided the results under two heads and comparison of the tables does not indicate any consistent variation in the total solids; but there does exist a remarkably striking uniformity with regard to the lower ash in the tissue fluids of spiked leaf. In the case of the Uttarahalli area, the average ash of healthy leaf fluid is about 30 per cent. higher, while the

corresponding figure for Ragihalli is 75 per cent. The differences in this respect are more pronounced in the Ragihalli samples.

Table III gives the values for nitrogen, phosphorus pentoxide and calcium, calculated in milligrams per 100 c.c. of the tissue fluid. Nitrogen is high in the case of the tissue fluids from spiked leaf, while calcium is low. The mean value for nitrogen in healthy leaf is 367·9 while that for spiked leaf is half as much again. Calcium in healthy leaf is nearly four times that of the spiked leaf fluid in the Uttarahalli area. In the Ragihalli area, the mean nitrogen value of spiked leaf is a little more than twice that of healthy leaf fluid, while the calcium value for spike is half that of healthy. The phosphorus pentoxide values by themselves are not very significant, but when combined with other values, as will be shown in a later communication, the results attain great importance.

Tables IV *A* and *B* give the direct reducing sugars, as well as sucrose and maltose, in 100 c.c. of the tissue fluids. It is seen that in most of the cases, there is higher concentration of these in the diseased tissues.

SUMMARY.

The tissue fluids derived from the healthy and diseased sandals have been examined for their content of total solids, ash, nitrogen, phosphorus, calcium and sugars.

A lower ash, a higher nitrogen, a lower calcium, a higher maltose content and a higher content of reducing sugars characterise the tissue fluids of the spiked leaf.

Our grateful thanks are due to Prof. R. V. Norris for his many helpful criticisms.

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III.—CONTRIBUTIONS TO THE STUDY OF SPIKE-DISEASE OF SANDAL (*SANTALUM ALBUM*, LINN.). PART IX.

Chemical composition of tissue fluids from the stem.

By M. Sreenivasaya and B. N. Sastri.

The tissue fluids from healthy and diseased sandal stems after expression in the usual way (*J. Indian Inst. Sci.*, 1928, **11A**, 23) were centrifuged at 3,000 r. p. m. and the respective centrifugates examined for their total nitrogen, total solids, ash, phosphorus and calcium. The results have been tabulated in the three following tables.

TABLE I A.

UTTARAHALLI AREA.

Yield of tissue fluid from stems (Uttarahalli).

Date	Percentage yield on weight of green material	
	Healthy	Spiked
30-4-28	28·9	29·9
7-5-28	33·0	27·5
22-6-28	25·3	24·2
Mean ...	29·1	27·2

TABLE I B.

(RAGIHALLI).

Date	Healthy	Spiked
8-7-27	23·0	13·0
22-7-27	19·8	12·9
29-7-27	15·1	17·0
15-5-28	12·6	16·7
Mean ...	17·6	14·9

TABLE II A.

Grams per 100 c.c. of tissue fluid (Uttarahalli).

Date	Healthy			Spiked		
	Total solids	Ash	Per cent. of ash in solids	Total solids	Ash	Per cent of ash in solids
5—8—27	11·88	0·71	5·99	6·46	1·40	21·60
27-10—27	11·77	1·22	10·38	11·77	1·63	13·83
9—1—28	15·30	1·26	8·23	9·93	1·63	16·41
11—4—28	9·58	1·30	13·56	8·94	1·84	20·58
20—4—28	8·54	1·23	14·39	8·78	1·89	21·57
30—4—28	7·87	1·05	13·31	9·35	2·13	22·74
7—5—28	7·98	1·29	16·15	11·08	2·60	23·47
22—5—28	6·83	0·93	13·64	7·86	1·93	24·56
8—6—28	10·73	1·52	14·12	12·71	3·18	25·04
22—6—28	10·40	1·40	12·47	9·27	2·16	23·34
Mean ...	...	1·19	...	...	2·04	...
Standard deviation ...	...	± 0·22	...	...	± 0·49	...

TABLE II B.

Ragihalli Area.

Grams per 100 c.c. of tissue fluid (Ragihalli).

Date	Healthy			Spiked		
	Total solids	Ash	Per cent. of ash in solids	Total solids	Ash	Per cent. of ash in solids
8—7—27	10·75	0·70	6·51	11·30	0·38	3·37
22—7—27	16·33	0·70	4·31	13·79	0·83	6·02
29—7—27	14·41	1·01	6·98	21·52	0·97	4·51
14-10—27	13·78	1·04	7·57	14·42	0·97	6·70
7-11—27	11·18	1·08	9·69	13·91	0·99	7·17
23-11—27	12·16	2·00	16·44	14·70	1·60	10·88
19-12—27	13·59	1·21	8·90	13·54	1·18	8·71
15—5—28	12·58	0·76	6·08	13·81	1·92	13·86
15—6—28	15·59	0·83	5·33	13·98	1·23	8·81
Mean ...	...	1·04	...	...	1·12	...
Standard deviation ...	...	±0·39	...	...	±0·41	...

TABLE III A.

Milligrams per 100 c.c. of tissue fluid (Uttarahalli).

Date	Healthy			Spiked		
	Total N	P_2O_5	Ca	Total N	P_2O_5	Ca
5- 8-27	...	92·0	136·0	...	99·6	128·0
15- 9-27	145 0	101·8	144·0	258·5	184 0	117·0
27-10-27	171·0	58·0	125·0	481·6	164·0	125·0
9- 1-28	247·8	33·0	163·0	415·6	77·0	129·0
11- 4-28	239·6	130·2	...	365·1	176·7	...
20- 4-28	281·1	168·4	36·0	398·0	194·2	90·0
30- 4-28	378·3	223·6	72·0	467·2	321·2	132·0
7- 5-28	270·1	148·9	62·0	280·2	237·6	116·0
22- 5-28	198·8	92·6	64·0	375·4	150·0	74·0
8- 6-28	262·6	108·1	80·6	591·8	114·9	101·6
22- 6-28	214·5	86·2	82·1	298·6	94·3	126·3
Mean ...	240·8	112·9	96·5	393·2	164·7	113·9
Standard deviation ...	± 61·9	± 50·4	± 40·0	± 96·9	± 67·9	± 18·3

TABLE III B.

Milligrams per 100 c.c. of tissue fluid (Ragihalli).

Date	Healthy			Spiked		
	Total N	P_2O_5	Ca	Total N	P_2O_5	Ca
8- 7-27	72·5	...	...	...	...	...
11- 8-27	...	45·6	103·0	...	68·1	89·0
14-10-27	95·0	41·0	133·0	162·0	66·0	129·0
7-11-27	96·3	109·3	121·0	147·3	153·1	114·0
23-11-27	153·6	68·0	94·0	176·8	35·0	94 0
19-12-27	128·0	7·0 (?)	104·0	214·0	32·0	82·0
15- 5-28	...	90·9	29·0	...	76·9	60·8
15- 6-28	188·2	78·8	77·5	254·7	100·1	64·5
Mean ...	122·3	72·3	94·5	190·9	75·9	90·5
Standard deviation ...	± 39·3	± 24·0	± 31·4	± 38·8	± 38·3	± 22·9

TABLE IV A.

UTTARAHALLI AREA.

Milligrams of calcium per 100 c.c. of tissue fluid
(Uttarahalli: healthy)

Date	Leaf	Stem
15—9—27	165·0	144·0
20—4—28	750·0	36·0
30—4—28	634·0	72·0
7—5—28	668·0	62·0
22—5—28	558·0	64·0
8—6—28	940·2	80·6
22—6—28	540·4	82·1
Mean	615·1	77·2
Standard deviation ...	±219·9	± 30·7

TABLE IV B.

(Uttarahalli: diseased).

Date	Leaf	Stem
15—9—27	107·5	117·0
20—4—28	154·0	90·0
30—4—28	154·0	132·0
7—5—28	118·0	116·0
22—5—28	154·0	74·0
8—6—28	120·0	101·6
22—6—28	96·8	126·3
Mean	129·0	108·1
Standard deviation ...	± 22·6	± 19·1

Discussion of Results.

Tables I A and B give the yield of tissue fluids from stems. It has been observed that the yield is lower in the case of diseased stems and progressively decreases with the onset of the disease. The root ends and the haustorial connections are damaged by the disease, so that the absorption of water and nutrients from the soil solution is affected. The heavy accumulation of starch in the conducting tracts of the stem renders free passage of the sap extremely difficult. This can be experimentally demonstrated by selecting two four-inch lengths of twig having the same diameter, one from the healthy and the other from the diseased sandal and sucking water through them under a known negative pressure. It will be found that, while water can easily be sucked through the healthy twig, the diseased offers very great resistance.

Tables II A and B give the total solids, ash in grams per 100 c.c. of the tissue fluid and also the percentage of ash calculated on the weight of the total solids. In the case of the diseased stem fluid, the ash is distinctly higher for the Uttarahalli area. The corresponding average value in II B also shows a slight increase, but is not significant. It is however clear that the ash is not lower in the diseased condition, as was shown to be the case in the instance of the tissue fluids from diseased leaves.

Tables III A and B give the nitrogen, phosphorus pentoxide and calcium values expressed as milligrams per 100 c.c. of tissue fluid. Nitrogen figures are uniformly higher in the diseased condition, and in the majority of instances the phosphorus values are distinctly higher in the diseased state. The calcium content of the diseased tissue fluid is in many cases higher and in fact the average value of calcium for the Uttarahalli area is about 17 per cent. higher. At any rate, the distinctly lower calcium content which characterises the diseased leaf tissue fluid, is not to be found in the case of the diseased stem tissue.

The study of gradients of concentration of the several nutrient elements from the root to the growing tips will throw light on the nature of the distribution. In the case of the healthy sandal, it will be observed from Table IV A that there is a great difference between the calcium concentrations of the leaf and stem tissue fluids in the healthy state.

The average value for the stem fluid is only one-tenth of the value for the leaf. In the diseased condition, however, the calcium concentration of the leaf tends to fall to that of the stem and in

advanced stages of the disease the concentration may fall far below that level (see Table IV B.)

Some investigators have found that pine trees grown in quartz sand and fed by a synthetic culture solution lacking calcium, exhibited irregularities in the transportation of starch, and a diminution in the size of the several organs (Loew, *U. S. Dept. of Agric. Bureau of Plant Industry*, 1903, Bull. No. 45). The low calcium concentration in the leaf, the main centre of photosynthetic activity, probably stimulates formation of starch, which in turn accumulates in the tissues of the diseased sandal.

SUMMARY.

The tissue fluids of healthy and diseased stems of sandal have been examined for total solids, total nitrogen, ash, phosphorus and calcium.

Nitrogen and phosphorus concentration of the tissue fluids from diseased stems is higher as compared with the values for the healthy stems, but the calcium content in the diseased state is higher.

A high gradient of calcium concentration exists from the stem towards the leaf which tends to flatten as the disease advances. It is suggested that the rapid transport of calcium through the stem is impaired with the incidence of spike; consequently, the leaf does not receive its adequate share of the calcium which, as shown by previous investigators, prevents abnormalities in the translocation of starch.

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IV.—NOTE ON THE STARCH-LIQUEFYING ACTION OF SANDAL LEAF EXTRACTS.

By B. N. Sastri.

It is well known that the spike-disease brings about a heavy accumulation of starch in the diseased tissues. It has also been found that the diastatic or sugar-producing capacity of the leaf extracts and tissue fluids in the diseased state is higher than that of the healthy. It was suspected that there might exist in the two extracts a varying proportion of the two well-known diastatic components, the liquefier and the saccharifier. The abnormal accumulation of starch suggests that in the diseased condition there is poor translocation of the carbohydrate, possibly due to a deficient proportion of the liquefying fraction. Experiments have been described to determine the proportion of the two enzyme components in the leaf extracts.

Potato starch paste (25 c.c. of 2 per cent.) was treated with 2 c.c. of the tissue fluids, expressed from leaves as described in a previous paper (*J. Indian Inst. Sci.*, 1928, **11A**, 24); the mixture, with about 0·5 c.c. toluene, was incubated at 30° for 20 hours, and 20 c.c. then added to 120 c.c. of 95 per cent. alcohol. The precipitate was filtered on a prepared Gooch crucible, previously dried and weighed, and was washed first with 95 per cent. alcohol, then with ether and dried in a steam oven to constant weight. The sugars in the filtrate were estimated by Bertrand's method, after removing the alcohol by evaporation and treatment with dialysed iron. The following table gives the results of estimation, the usual controls being run; difference in the weights of precipitate between control and experimental gives the weight of starch liquefied.

Hydrolysis of Potato starch by leaf extracts (Uttarahalli).

Milligrams of	8–8–28		23–8–28	
	Healthy	Spiked	Healthy	Spiked
Starch liquefied ...	93·1	225·9	52·0	162·0
Maltose produced ...	46·5	172·5	26·1	120·3

It is clear from the table that for equal weights of sugar produced, more of starch is liquefied by the healthy leaf extract than by the

spiked. Thus calculating the amount of starch liquefied for 100 mgs. of sugar produced, it is seen that 200·2 mgs. of starch have been liquefied by the healthy leaf extract (August 8, 1928) and 130·9 mgs. of starch by the spiked extract. The corresponding figures for 100 mgs. of sugar from the sample of August 23, 1928 are 199·2 mgs. and 134·6 mgs. Thus the healthy leaf extract is a more efficient liquefier than the spiked leaf extract. The results have also been confirmed by measuring the fall in viscosity of the reaction mixture containing potato starch and leaf extracts; this is more rapid in the case of mixtures containing healthy leaf extracts than in those containing spiked leaf extracts.

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STUDIES ON SOIL *ACTINOMYCES*. PART III. STANDARDISATION OF A PLATE METHOD OF COUNTING SOIL *ACTINOMYCES*.

By M. Ganesha Rao and V. Subrahmanyan.

It was shown in the previous communication (*J. Indian Inst. Sci.*, 1929, **12 A**, 57) that in the normal soil *Actinomyces* are found almost exclusively in the form of conidia, and that, when they occur vegetatively, as they do in some rare cases, their mycelia are largely present on undecomposed plant residues and similar forms of organic matter which are generally removed during sampling, and not on the soil itself. Since the conidia resemble many of the commoner forms of bacteria in shape and size, no direct method of counting them, stained or otherwise, will be feasible. The plate method alone can provide some information regarding the numbers and distribution of *Actinomyces* in soil.

The plate method will not, however, be satisfactory unless it fulfils the following conditions:—(I) The count medium should (*a*) be composed of materials well-defined in composition and character, (*b*) be readily reproducible, (*c*) contain minimal quantities of essential nutrients and bring out the largest numbers without encouraging any abnormal vegetative growth, (*d*) bring into relief the cultural characteristics distinguishing different species of *Actinomyces* not only from other microflora, but also from each other, and (*e*) discourage growth of other organisms, particularly those of spreading bacteria and fungi. (II) Details relating to the making of plates should be readily applicable not only to all types and conditions of soils but also to different species of *Actinomyces* present in them. (III) Parallel plates should provide counts which are (*a*) within limited range of variation from each other, and (*b*) distributed in a well-defined manner over the plates.

Though numerous media have been used by different workers, none of them has yet been tested for its fitness to be used as the standard medium for counting *Actinomyces*. The earlier workers used exclusively beef extract media solidified with agar or gelatin. Among the later workers Krainsky (*Zentr. Bakt.*, 1914, II, **41**, 649) found these unsatisfactory, preferring calcium malate agar and similar synthetic media. Conn (*J. Bact.*, 1916, **1**, 197) used an agar medium containing sodium asparaginate and glycerol. Waksman and Curtis (*Soil Sci.*, 1916, **1**, 99) noted that a medium containing albumen was efficient for

counting *Actinomyces* together with bacteria. As a result of applying such diverse media, counts obtained by different workers, though useful by themselves, were not comparable with each other.

The object of the present investigation was to develop a standard method for counting soil *Actinomyces* which would satisfy the conditions set forth already.

EXPERIMENTAL.

Preliminary trials with the commoner plate and culture media were carried out with a view to (*a*) determining the extent to which each could approach the ideal, and (*b*) obtaining a line for further improvement on the most promising among them. The media tried were: (1) Modified albumen agar (Waksman and Curtis, *loc. cit.*), (2) Glucose agar (Krainsky, *loc. cit.*), (3) Soil extract agar (Fischer: Waksman, *Principles of Soil Microbiology*, 1927, pp. 15, 16 and 291), (4) Casein agar (*ibid.*), (5) Nutrient agar with 1 per cent. glycerol, (6) Synthetic agar (Lipman and Brown, *ibid.*), (7) Malate, glycerol agar (Krainsky, *loc. cit.*), (8) Sodium asparaginate glycerol agar (Conn, *loc. cit.*), (9) Urea, ammonium nitrate agar (Cook, *Soil Sci.*, 1916, **I**, 153), (10) Starch agar, (11) Citrate, glycerol agar (Conn, *N. Y. Agric. Exp. Sta. Tech. Bull*, 60, 1917) and (12) Czapek's agar which, with 10, is also specified in Waksman's book. Four specimens of soil representing the types obtained in different parts of India were used for the platings. They were surface samples (0–9″ depth) of (A) black cotton soil from Hyderabad, Deccan, (B) alkaline, wheat-growing soil from Gujranwala District, Punjab, (C) peaty soil from Travancore, South India and (D) low-lying paddy land from Dacca, Bengal.

The technique adopted for plating in the preliminary and most of the subsequent trials was as follows:—After being air-dried, powdered and passed through the millimeter mesh sieve, 10 gms. of the soil were shaken vigorously for 5 minutes with 100 c.c. of sterile tap-water in a stoppered flask, 10 c.c. of the suspension removed before settling and transferred to a second flask containing 90 c.c. of sterile tap-water. The second suspension was shaken for one minute and 10 c.c. transferred to a third flask containing 90 c.c. of sterile tap-water. After being shaken again for one minute, 1 c.c. of this third suspension was transferred to 90 c.c. of sterile tap-water, and 1 c.c. portions of this fourth suspension used with frequent shaking for the platings, which were carried out in quadruplicate. Incubations were at 25–30°, and the final counts taken at the end of a fortnight. In the preliminary trials observations and counts were made once in two days to compare the relative growths of the different forms of microflora appearing on them.

Before discussing the results, it may be mentioned that most of the above-mentioned media were slightly acid, though in a few cases the reactions were adjusted to be neutral. With the exception of medium 3 all contained at least 1 per cent. of glucose, sucrose, or glycerol as the main carbon nutrient. Media 1, 2, 6, 7, 9 and 11 contained less than 0·05 per cent. of nitrogenous nutrients which were egg albumen, asparagine, peptone, ammonium chloride (7 and 11), urea and ammonium nitrate respectively: their P_H as measured colorimetrically (Medalia, *J. Bact.*, 1920, **6**, 441) was also varied, being 6·4, 5·3, 5·4 and 7·0 (7, 9 and 11) respectively. The nitrogen content of 3 was very low and of indeterminate composition; the P_H of that medium was 6·2. Media 4, 8, 10 and 12 contained between 0·1 and 0·2 per cent. of casein, dihydrogen ammonium phosphate, ammonium sulphate and sodium nitrate respectively; their P_H was 6·2, 6·8, 7·0 and 6·4 respectively. Medium 5 was richest in nitrogen and contained 1 per cent. peptone. The mineral contents of the different media did not vary appreciably from each other.

In the present and the subsequent trials, *Actinomyces* appearing on the plates were distinguished from other organisms by the following cultural characteristics:—(I) Formation of (*a*) round or very nearly round colonies, which spread only slowly, (*b*) aerial mycelia which under favourable conditions rose above the medium-surface and were composed of very fragile hyphæ, disrupted by gentle prodding with the needle, (*c*) concentric 'fairy-rings' composed almost exclusively of reproductive hyphæ and conidia, (*d*) the characteristic earthy odour detected in most cases after even the merest touch of the colony with a sterile needle, (*e*) variegated colours formed by either or both substrate growths and aerial hyphæ. (II) Microscopic appearance of the colony edges which, being composed of thin, close-packed hyphæ, distinguished *Actinomyces* readily from all other forms of micro-organisms. Repeated trials showed that even when none of the characteristics mentioned under I were exhibited, *Actinomyces* never failed to present the characteristic edge appearance (II) at any stage during growth.

The observations showed that although on most of the plates *Actinomyces* began appearing before the end of one week, they could not, in the earlier stages, be readily distinguished from bacteria except by microscopic examination. Growths on medium 10 were, however, prominent from the beginning and exhibited all the characteristics mentioned already. The total numbers of micro-organisms including *Actinomyces*, counted at two-day intervals, presented a confusing mass of data. From the three following typical sets of counts (Table I) taken after one-week's incubation for two soils, it will appear that,

TABLE I.

Medium No.	Colonies per gm. of soil (millions)	
	Soils	
	A	C
2	2·0	1·1
4	3·7	3 9
11	1·9	6·2

depending on the medium used, Soil C contained numbers which were (*a*) less than, (*b*) equal to, and (*c*) much greater than those present in Soil A. All the data were, therefore, discarded.

It was also observed that the total numbers did not increase steadily with appearance of new colonies from time to time; the counts rose and fell spasmodically. Microscopic examinations of circlets locating colonies that had been previously observed showed that many did not contain even traces of the original growths, thereby indicating that the older colonies steadily disappeared while new ones came up. The study of conditions leading to such disappearances will form the subject of a later communication.

When the final counts were made, it was observed that media 4, 5 and 12 encouraged growths of numerous fungi and bacteria which spread rapidly and almost completely covered the *Actinomyces* that appeared. No more than one or two poorly formed colonies of *Actinomyces* appeared on 3 which, obviously, lacked the minimal requirements of the organisms. Media 7, 8 and 11 brought out large numbers of spreading bacteria, but comparatively few *Actinomyces*, which made no satisfactory growth.

Media 1, 2, 6 and 9 carried fewer bacteria, but the *Actinomyces* showed no marked cultural characteristics differentiating them readily from other organisms. Medium 10 proved to be the most satisfactory and brought out a fairly large number of colonies with all their cultural characteristics: bacteria and fungi made very poor growths, and at no time interfered with the development of *Actinomyces*. The final counts (Table II) of *Actinomyces* taken at the end of the fortnight were rather low, indicating thereby that the soils were not comparable with those for which some previous workers (e.g., Conn, *loc. cit.*) using the same media obtained higher counts.

TABLE II.

Medium	*Actinomyces* per gm. of soil (100,000's)			
	Soils			
	A	B	C	D
1	9	11	8	10
2	10	8	4	5
3	*	*	*	*
4	16	10	9	14
5	*	*	*	*
6	5	10	7	9
7	7	7	8	6
8	7	7	8	6
9	7	8	10	7
10	12	15	13	7
11	8	14	11	5
12	7	8	6	9

The starch medium, in spite of its advantages, was difficult to work with, because it contained large amounts of (*a*) potato starch which made it sticky and (*b*) calcium carbonate which rendered it opaque.

Effect of altering the proportions of minerals and nitrogen in the starch medium.—To determine whether (1) the carbonate can be replaced by a soluble salt of calcium and (2) the quantities of nitrogen and minerals can be reduced to a minimum, platings were made using a medium (II) composed (in grams) of starch (potato), 10; HK_2PO_4, 0·5 $(NH_4)_2SO_4$, 0·2; $MgSO_4 7H_2O$, 0.2; $CaCl_2$, 0·1; $Fe_2(SO_4)_3$ a trace, in distilled water, 1,000 c.c. with washed agar, 20 grams, the P_H being adjusted to 7·2. The counts, as compared with those on the original starch medium (1), are shown below.

The deviations being alternately in favour of media I and II, it may be inferred that the change in composition had not altered significantly the counts of *Actinomyces*. Medium II was the clearer, but suffered more from spread of fungi.

* Practically no colonies, or uncountable owing to overgrowth of fungi.

TABLE III.

Starch medium	*Actinomyces* per gm. of soil (100,000's)			
	Soils			
	A	B	C	D
I	12	15	13	7
II	14	14	12	10

Varying the source of nitrogen.—To determine whether (1) starch was superior as a source of carbon to glucose which had been used by several of the earlier workers and (2) the use of other nitrogenous compounds in place of ammonium sulphate used in the previous trials could bring out larger numbers of *Actinomyces* and check more effectively the spread of fungi, platings were carried out with media containing the same proportions of minerals as II, but differing from it in containing other nitrogen compounds in place of ammonium sulphate together with 1 per cent. each of glucose (Series III) and potato starch (Series IV) respectively. Nitrogen was supplied in the forms of asparagine, 0·30 gm. (IIIa and IVa); ammonium chloride, 0·17 gm. (IIIb and IVb); sodium nitrate, 0·30 gm. (IIIc and IVc) and urea, 0·10 gm. (IIId and IVd) respectively added to every litre of medium. Control experiments were also carried out using a medium containing only starch and minerals. Soil B which gave consistent numbers in previous trials was used for plating. The counts (Table IV) showed that, although series IV did not give significantly better counts than series III, *Actinomyces* appearing on the former made very much better growths and were almost entirely free from

TABLE IV.

Actinomyces per gm. of soil (100,000's)									
Glucose					Starch				
III (control)	IIIa	IIIb	IIIc	IIId	IV (control)	IVa	IVb	IVc	IVd
9	10	8	10	9	11	13	12	14	12

spreading bacteria and fungi. The colonies appearing on control plates were poorly formed, indicating that the nitrogen requirements of

Actinomyces, though small, should nevertheless be satisfied. The plates containing asparagine were the cleanest: those containing urea were marred by spread of fungi.

Effect of reducing the quantities of nitrogen and starch.—To ascertain whether (1) the supply of nitrogen can be further reduced, (2) mixtures of nitrogenous compounds will prove more effective than single substances and (3) the quantity of starch can be lowered, trials were carried out using media (Va–Vd) containing the same amounts of minerals as in the previous trials, together with asparagine, 0·05 gm. and (Va) sodium nitrate, 0·05 gm. and potato starch, 10 gms., (Vb) ammonium chloride, 0·05 gm. and potato starch, 10 gms., (Vc) sodium nitrate, 0·05 gm. and potato starch, 2 gms., and (Vd) ammonium chloride, 0·05 gm. and potato starch, 2 gms.—per litre of medium in each case.

It was observed that reduction in the quantity of starch rendered the media easier to handle and markedly increased the counts (Table V).

TABLE V.

Soil	*Actinomyces* per gm. of soil (100,000's)			
	Va	Vb	Vc	Vd
B	15	12	22	16

It was also noticed that plates containing asparagine and nitrate (Vc) not only carried the largest numbers of colonies but were also the cleanest. Media containing ammonium chloride encouraged growth of fungi.

Starches from different sources.—Potato starch which was used in the previous trials was not satisfactory because it (1) rendered the medium cloudy, and (2) was not uniform in quality. To ascertain whether more satisfactory counts can be obtained by using starches from other sources, trials were carried out with media (VIa–VId), identical with Vc except that rice (VIa), maize (VIb), wheat (VIc) and soluble (B.D.H., A.R.) starches (VId) were used in place of that from potato. Soils A and B were used for the platings. The counts are given in Table VI.

TABLE VI.

Soil	*Actinomyces* per gm. of soil (100,000's)				
	Vc	VIa	VIb	VIc	VId
A	29	11	21	19	31
B	22	9	18	14	23

It was observed that growths of *Actinomyces* on rice-starch were poor, while those on maize and wheat-starch were largely overgrown with fungi. The soluble starch medium, though not superior to that of potato starch in the numbers, was otherwise more satisfactory because of its clarity and greater efficiency in checking spread of fungi. Soluble starch possessed also the advantage of being a standard product of reproducible quality.

Effect of changing the quantity of soluble starch.—To ascertain whether the proportion of soluble starch can be altered with advantage, trials were carried out with media identical with VId but containing different amounts of soluble starch. They were, (VIIa) only minerals and nitrogen, without any starch, as control: VIIb–VIIe containing 0·05, 0·1, 0·2, and 0·3 per cent. respectively of soluble starch: and VIIf only 0·2 per cent. starch, without minerals or nitrogen, as control. Soils A, B, C and D were used. The counts are given in Table VII.

TABLE VII.

Medium	*Actinomyces* per gm. of soil (100,000's)			
	Soils			
VII	A	B	C	D
a	10	7	6	3
b	7	7	...	3
c	9	8	5	4
d	28	22	25	10
e	16	36	17	5
f	3	10	9	2

It was observed that colonies appearing on media VIIa, VIIb, VIIc and VIIf were not only numerically small, but also poorly formed, indicating that the media did not supply the minimal requirements of the organisms for making satisfactory growth. Counts on VIIe were less consistent than those on VIId.

It is interesting to note that there was much greater response from *Actinomyces* to (1) starch from potato rather than to that from any of the grains, (2) one particular concentration of soluble starch than to others. A study of the causes leading to such observation is being made and will form the subject of a later communication.

Replacing soluble starch by other carbohydrates.—Platings were made in media identical with VIId but containing 0·2 per cent. each of maltose (VIIIa), lactose (VIIIb), dextrin (VIIIc), inulin (VIIId), glycogen (VIIIe), fructose (VIIIf) and xylose (VIIIg) in place of soluble starch.

The poor counts that were obtained (Table VIII), together with the observation that the colonies were meagrely formed, showed that none of the carbohydrates was favourable to the development of

TABLE VIII.

Medium VIII	*Actinomyces* per gram of soil (100,000's)			
	Soils			
	A	B	C	D
a	10·8	3·2	*	1·2
b	10·2	6·4	9·6	4·8
c	8·2	10·4	*	1·4
d	4·2	3·4	*	5·2
e	10·2	7·8	1·4	9·4
f	*	2·2	*	1·8
g	*	8·2	*	1·4

* No colonies, or completely overgrown with fungi.

Actinomyces. Profuse growths of bacteria and fungi occurring on the plates showed, on the other hand, that the media were suited only to such organisms.

The foregoing data confirm the previous observation (*vide* Table I) that counts on different media though for the same soils did not follow any order. They suggested that formation of colonies on count media were determined not only by the numbers of living cells present in the soils but also by the individual response of the different organisms to the nutrients provided by the media. The latter property in the case of soil organisms would depend on the previous conditions such as manuring and cropping of the soils concerned.

Effect of increasing the asparagine and nitrate.—Platings were made on media identical with VIId but containing asparagine and nitrate (IXa), 0·1 gm. each; (IXb) 0·2 gm. each; (IXc) 0·5 gm. each respectively per litre of medium. The counts (Table IX) showed that

TABLE IX.

Soil	*Actinomyces* per gm. of soil (100,000's)			
	VIId	IXa	IXb	IXc
B	22	14	13	8

in addition to giving lower counts, the media containing larger amounts of nitrogen encouraged growths of spreading bacteria and fungi. To suppress spreading colonies it would therefore be necessary not only to choose the proper forms of carbon and nitrogen, but also to maintain their quantities at the minimum.

Altering the mineral composition of the medium.—To find whether (1) all the mineral and nitrogen compounds used in the previous trials were essential, (2) some of the ingredients can be replaced advantageously by allied compounds, and (3) addition of certain new substances can lead to marked alteration in the number of colonies, trials were conducted with media which were the same as VII*d*, except in the following respects:—Xa, without dipotassium phosphate; Xb, without asparagine; Xc, without sodium nitrate; Xd, without ferric sulphate; Xe, without magnesium sulphate; Xf, without calcium chloride; Xg, with potassium nitrate in place of the sodium salt; Xh, with disodium phosphate in place of the dipotassium salt; Xi, together with 0·01 per cent. zinc chloride; Xj, together with 0·01 per cent. aluminium acetate. P_H was adjusted to 7·4 in all cases.

The zinc salt was added to determine whether it had a stimulating effect similar to that it has generally on fungi. Since free aluminium is known to be present in many acid soils which do not encourage the

added to determine whether it had any inhibitory action. The platings were carried out with soils A, B, C and D. The counts (Table X) showed that none of the media was as satisfactory as VIId.

TABLE X.

Medium X	*Actinomyces* per gram of soil (100,000's)			
	Soils			
	A	B	C	D
a	6·4	16·4	15·2	6·2
b	13·6	15·8	13·2	8·8
c	14·8	18·8	14·6	5·4
d	15·0	19·4	9·8	6·6
e	6·8	16·6	17·0	7·8
f	10·4	17·6	13·2	13·4
g	5·8	17·8	12·6	5·8
h	13·8	20·0	18·2	4·4
i	8·2	17 0	18·4	1·8
j	3·8	14·8	12·2	9·8

It is interesting to observe that the counts for soils B and D were affected only slightly by alteration in mineral-supply and those for C to a slightly greater extent, while those for A were considerably lowered in every case. The varied response given by *Actinomyces* from different soils suggested that their previous conditions should have determined such behaviour. It is thus possible that the organisms from soils B and D had sufficient reserves of the essential mineral nutrients while those from A had not, so that when plated on media lacking one or the other of the salts, the organisms from B and D did not miss them while those from A did.

The counts on media Xa to Xj show the importance of every one of the components of medium VIId in helping to bring out a satisfactory number of colonies. The data for Xg and Xh show that sodium and potassium could not advantageously replace each other: those for Xi and Xj show that addition of zinc chloride and aluminium acetate led to no improvement in the counts.

It was also observed that the plates unsatisfactory with regard to counts of *Actinomyces*, generally carried larger numbers of fungi and spreading bacteria than the normal ones.

Reaction of the count medium.—To determine the most favourable reaction, platings were made on media which had the same composition as VIId, with the P_H adjusted as follows:—XIa, 6·2; XIb, 6·6; XIc, 7·0; XId, 7·4; XIe, 7·8 and XIf, 8·2. Medalia's colorimetric standards (*loc. cit.*) were used. The counts given in Table XI showed that the best were obtained only when the media were slightly alkaline.

TABLE XI.

Soil	*Actinomyces* per gm. of soil (100,000's)					
	XIa	XIb	XIc	XId	XIe	XIf
A	19	21	27	30	29	21
B	13	17	24	25	26	26

Although XId and XIe gave almost identical counts, the colonies on the former were better formed than those on the latter. P_H 7·4 was inferred to be the most favourable.

It was observed that above P_H 8·0, the colonies dwindled in size, while below P_H 7·0, though the numbers decreased, the colonies were larger and better-formed than the normal ones. The observation suggested that (1) *Actinomyces* were not tolerant of more than slight alkalinity and (2) certain species throve better in acid, than neutral or alkaline conditions. *A. acidophilus* (*Soil Sci.*, 1928, **25**, 225) is probably one of these.

Preparation of the Standard Medium.

The foregoing trials having shown that the best counts of *Actinomyces* were obtained with medium VIId, attempts were made to standardise the conditions for its preparation. On mixing the components in different ways, it was observed that potassium phosphate led, on boiling, to precipitation of magnesium, calcium and iron which were removed on filtering. Previous addition of starch in solution, however, protected the salts from coagulation and preserved them in a finely divided state which passed the filter readily. The protective action was greatly increased by adding the necessary amount of molten agar along with starch.

The following method was found to be satisfactory for preparing the medium:—Standard soluble starch (2 gms.) was made into a uniform paste with about 25 c.c. of distilled water, the volume being increased to about 250 c.c. and the mixture autoclaved for 10 minutes at a pressure of 5 lbs. to gelatinise the starch and to bring it to even dispersion; 500 c.c. of filtered, 4 per cent. washed agar were melted at the same time and added to the hot starch solution with frequent stirring. Dipotassium phosphate (50 c.c. of a 1·0 per cent. solution) was then added slowly to the starch-agar mixture, with frequent stirring. The other ingredients of the medium (in grams) were $MgSO_4\ 7H_2O$, 0·2; $CaCl_2$, 0·05; $NaNO_3$, 0·05; asparagine, 0·05; and $Fe_2\ (SO_4)_3$ (1 drop 1 per cent. solution); these were made up in a separate mixture from stock 1 per cent. solutions and added. The whole was added in small instalments to hot agar-starch-phosphate mixture with stirring and raised to boiling before adjusting the reaction by Medalia's method (*loc. cit.*).

STANDARDISATION OF PLATING DETAILS.

The previous trials showed that air-dry specimens passed through the millimeter-mesh sieve provided uniform dispersion of the soil particles and give fairly uniform counts. But it was not clear whether the tap-water used was a really satisfactory disperse medium. Plating trials were therefore carried out comparing tap-water with the saline solution containing 5 gms. NaCl and 1 gm. $MgSO_4$, 7 H_2O in 1000 c.c. of distilled water recommended for plating soil bacteria (Thornton *Ann. Appl. Biol.*, 1922, **9**, 241). The counts (Table XII) were very

TABLE XII.

Disperse medium	*Actinomyces* per gm. of soil (100,000's)			
	Soil			
	A	B	C	D
Tap-water	32	25	29	11
Saline solution ...	33	23	26	13

nearly the same for both the disperse media showing that the conidial form in which the *Actinomyces* were mostly present was not sensitive to the osmotic conditions of the dispersing fluid.

It was observed that shaking soil suspensions by hand for definite periods did not necessarily lead to even dispersion of the soil particles

because (1) depending on the strength and temperament of the worker the suspensions were well or ill shaken during the same period, (2) shaking vigorously and steadily during 5 minutes for every case, particularly for numerous platings, was exacting, (3) whatever the time spent, if the shaking during the last stages was not vigorous and the suspensions were not removed before settling could occur, the counts proved to be unsatisfactory.

Trials were therefore conducted to determine whether (1) the time of shaking could be conveniently reduced, and (2) shaking vigorously and continuously for a fixed number of times would be more efficacious than an indefinite rate for a definite period. The suspensions were removed quickly before settling. The details of the experiments together with the counts obtained are given in Table XIII.

TABLE XIII.

Mode of Shaking	*Actinomyces* per gm. of soil (100,000's)			
	Soils			
	A	B	C	D
I. Time—5 minutes ...	33·4	24·8	28·8	12·0
I. ,, —3 ,, ...	31·8	25·6	26·4	10·6
I. ,, —1 ,, ...	24·6	21·4	26·6	10·2
II. Number—200 ...	32·6	23·6	28·6	11·4
II. ,, —150 ...	32·0	24·4	27·8	11·8
II. ,, —100 ...	29·8	22·8	26·6	10·6

I. Slow with occasional stoppages: II. Vigorous non-stop.

The results showed that reduction in time of shaking to 3 minutes had not greatly affected the counts, while the further reduction to 1 minute had. Shaking vigorously for 200 or 150 times during about one minute gave results as good as those following less vigorous agitation during five minutes. It may be inferred that the vigour of shaking rather than the time occupied effected the dispersion of soil particles.

It was further observed that uniform counts could be obtained by shaking for only 100 times for the second and subsequent dilutions; not containing much solid matter, the suspensions became readily dispersed.

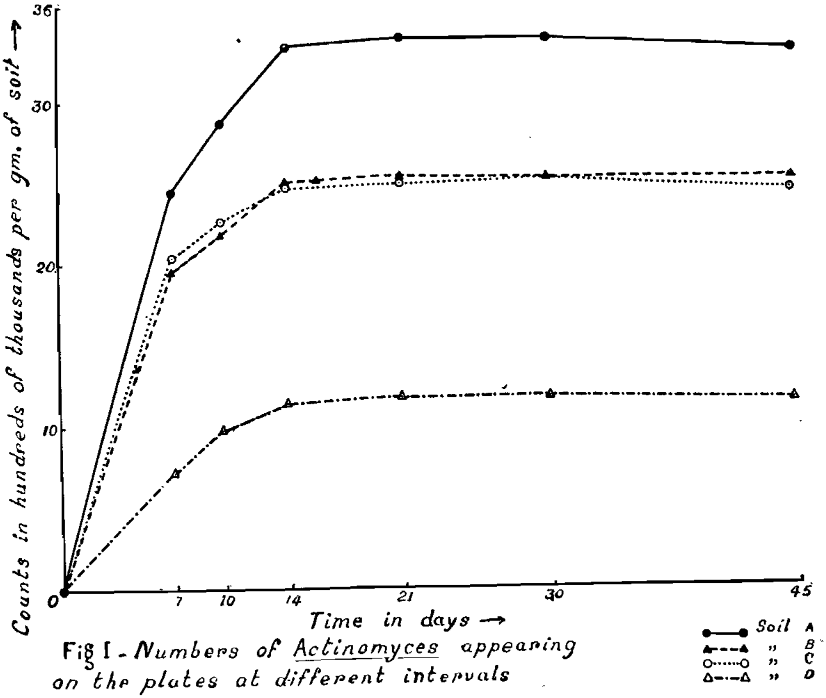

Fig I - Numbers of *Actinomyces* appearing on the plates at different intervals

Dilutions to be Employed.

Platings with soils from various localities showed that it was not always advantageous to adopt a dilution of 1 in 100,000. Counts (Table XIV) for soils from different parts of India and Ceylon showed that even 1 in 1,000 had to be employed in certain cases.

TABLE XIV.

Locality	Description	Crop raised	*Actinomyces* per gm. of soil (10,000's)
Hyderabad, Deccan	Black cotton ...	...	334
Gujranwala Dt., Punjab	Alkaline ...	Wheat ...	254
Travancore, S. India	Peaty ..	Paddy ...	234
Dacca, E. Bengal	Alluvial ...	Paddy ...	100
Bundelkhand, U. Provinces ...	...	...	216
Sholapur	Karl soil ...	...	95
Kandy, Ceylon	Reddish laterite ...	Tea ...	25
Mirphukas Farm, Sindh	Kalar soil ...	Fruit ...	68
Tellicherry, S. India	Red sandy loam ...	Coconut ...	4

The number of colonies of *Actinomyces* allowable on the plate depended to a large extent on the average size of the individual colonies and the other forms of microflora appearing at the same time, but under normal conditions 40 was found to be convenient. The best results were obtained by adjusting the dilution to give between 30 and 40 colonies per plate.

Period and Temperature of Incubation.

To ascertain whether incubation for a fortnight as in the previous trials was adequate, the platings were made on the standardised medium using soils A, B, C and D. The counts (Fig. 1) showed that (1) most of the colonies appeared simultaneously at the end of a week, and (2) subsequent increase in number was appreciable up to the end of a fortnight, but was insignificant in later stages.

Incubations at 37° showed that although colonies of *Actinomyces* appeared even at the end of about four days, they were more readily apt to be over-run by spreading organisms than at a lower temperature. It was also observed that variations of temperature between 25° and

30° did not appreciably affect the final counts. The same range of temperature is considered satisfactory for platings using the standard medium.

Method and Accuracy of Counting.

Since *Actinomyces* were always prominent by their size and cultural characteristics and comparatively small in numbers, it was found possible, throughout this investigation, to count all the colonies that appeared on the plates. A similar method is recommended for use with the standard medium.

Consistency of individual counts.—To learn whether, under technically perfect conditions, use of the standard medium can give consistent counts, platings of soils from different parts of India and Ceylon were made. Dilutions of 1 in 100,000 were adopted in all the cases. The counts (Table XV) showed that they were fairly uniform, indicating

TABLE XV.

No.	Locality	Description	Counts on individual plates				
			I	II	III	IV	V
1	Jacobad, Punjab ...	Govt. wheat farm ...	30	32	28	32	...
2	Nasik, Bombay ...	Garden soil ...	32	32	34	28	31
3	Gaya, Bihar ...	Waterlogged ...	4	6	4	5	5
4	Chirakkal, Madras ...	Sandy loam, Paddy ...	35	46	40	38	42
5	Ratnagiri, Bombay ...	... ...	17	15	12	14	...
6	Jaffna, Ceylon ...	Sandy, Coconut ...	14	13	11	11	12

that under ideal conditions, which could not be attained experimentally because of the errors inherent to the dilution process, they should have been identical.

Because of the small numbers involved, errors will in certain cases be highly magnified in spite of the uniformity of individual counts. Thus the standard errors of the averages from different sets of counts represented as percentages (Table XVI) were, (1) 3·1 ; (2) 3·2 ; (3) 7·8 ; (4) 6·2 ; (5) 7·2 ; and (6) 4·8 respectively, showing that, although the individual counts of sets (*c*) and (*e*) were much more consistent than those of (*d*), the errors of their averages were greater than those of the latter because of the smallness of their counts. As suggested already, the adjustment of dilution for such soils to give between 30 and 40 colonies per plate will greatly minimise the error.

Distribution of counts from random samples.—Knowledge of the exact manner of distribution of the colonies on parallel plates is essential before adopting a technique in the study of problems relating to soil *Actinomyces.* Thus, if it be known that under technically perfect conditions the parallel counts will vary within a small range from their mean, then a count from a similar sample of soil which deviates beyond the recognised range will denote a significant difference in biological condition from the first sample. If, on the other hand, individual counts from the same sample vary considerably from each other, the technique of counting will be useless to detect small changes in numbers.

In their study of the accuracy of the plate method of counting bacteria, Fisher, Thornton and Mackenzie (*Ann. Appl. Biol.*, 1922, **9**, 325) observed that the colonies were distributed in the manner of samples of the Poisson series. Thus, if m be the known average of a number of bacterial counts, then the frequencies with which individual counts can bear values 0, 1, 2,........, n, ...will be given theoretically by the series :—

$$e^{-m}\left(1, m, \frac{m^2}{2!}, \frac{m^3}{3!}, \ldots\ldots, \frac{m^n}{n!}, \ldots\ldots\right)$$

Given the average, the mode of distribution of the individual counts can be calculated and used for checking the accuracy of those actually obtained by counting.

Since the plate method of counting *Actinomyces* was similar in principle to that adopted for bacteria, it was thought that in a like manner, the individual counts would be distributed in the manner of samples of the Poisson series.

To verify this, platings were made using a number of samples from one single specimen of soil. The Chirakkal soil (20 gms.) used in the previous trial was mixed with 200 c.c. of sterile saline and thirteen sets of dilutions of 1 in 100 were prepared from the suspension, using sterile tap-water in seven and sterile saline in the remaining six cases as disperse media. Further dilutions to 1 in 100,000 were then prepared in the manner already described using sterile tap-water and saline respectively. They were then plated on the standard agar medium using 5 plates for each set. From the counts obtained after a fortnight's incubation two statistics were calculated from each set of data. They were (1) the mean, $\bar{x}$ and (2) the variance, V which was calculated according to formula,

$$V = \frac{1}{n-1} \cdot S(x - \bar{x})^2$$

where n represented the number of plates and $S(x-\bar{x})^2$ the sum of squares of deviations of individual data from the mean. The object of the calculation was to ascertain whether the values of V approximated to those of $\bar{x}$ as should be the case with samples of the Poisson series. The figures thus calculated (Table XVI) showed that in every one of

TABLE XVI.

Tap Water		Saline Solution	
$\bar{x}$	V	$\bar{x}$	V
38·6	9·3	38·0	5·0
40·2	3·7	39·2	6·7
38·5	2·3	40·2	3·5
36·6	3·3	39·4	10·3
39·2	5·7	38·4	4·5
38·8	5·7	39·2	11·2
38·8	6·7	...	...

the cases, the value of V was less than one-third of that of $\bar{x}$. If the counts approximated to samples of the Poisson series, the values of V should have ranged on either side of the corresponding values of $\bar{x}$. Since they were distinctly lower, it may be inferred that the counts of *Actinomyces* were distributed in the manner of a normal population.

A study of the various operations involved in the plating showed that since (1) the error of sampling—which, in the above case, had been avoided—should have been merely due to random selection from a normal population, (2) the dilutions were carried out carefully and involved only a small experimental error, (3) the colonies of *Actinomyces* appeared on the plates without interfering with each other or with the other organisms and (4) all the colonies were counted, the plating technique, as applied to the counting of *Actinomyces* involved no risk as observed in the cases of random samples of the Poisson series.

The essential difference between the techniques of counting *Actinomyces* and bacteria lies in the fact that, whereas in the former all the colonies appearing on the plates are counted, in the latter only those appearing on certain segments of the petri-dish are counted.

Although under ideal conditions, the bacteria should be distributed evenly over the plates the numbers appearing on individual segments of petri-dishes will be proportional to the areas covered by

them, yet it is not realised in practice and there is always the risk of their not settling in the required numbers over the particular areas that are counted.

In the course of his study of the distribution of yeast cells over the counting area of the hæmocytometer, it was observed by 'Student' (*Biometrika*, 1907, **5**, 351) that the numbers occurring on the individual squares were samples of the Poisson series. If with the same suspension of yeast cells, different sets of the hæmocytometric counts be made, the numbers of cells settling on individual squares in the different sets will be related to each other as samples of the Poisson series. A similar distribution was also observed (Fisher, Thornton and Mackenzie, *loc. cit.*) with regard to the settling of bacteria not only on the individual segments of one petri-dish, but also on those of a number of other dishes when all of them were plated from similar suspensions of bacteria. It being difficult, in practice, to count all yeast cells settling on hæmocytometer, or bacteria on petri-dish, the element of 'risk' arises, obviously, from the need to reckon totals by multiplying by the necessary factors the counts obtained for only a few representative squares or segments as the case may be. Since according to the proposed technique every one of the colonies of *Actinomyces* appearing on the plates was counted, the 'risk' was avoided and the numbers appearing on parallel plates were distributed in the manner of samples of the normal series.

Influence of medium composition on distribution.—Platings were made with the Chirakkal soil on soil extract agar (Fisher, cited from Waksman, *loc. cit.*), the details being the same as in the previous experiment. The values of $\bar{x}$ and V obtained for the different sets of counts (Table XVII) showed that (1) the averages ($\bar{x}$) were not consistent, particularly for suspensions in saline solution, and (2) the variations V exceeded those indicating accuracy sufficient to detect small

TABLE XVII.

Saline Solution		Tap-Water	
$\bar{x}$	V	$\bar{x}$	V
30·3	51·0	36·0	12·0
36·2	16·5	30·0	5·5
37·3	19·0	38·2	85·4
39·0	51·5	37·2	77·5
43·0	150·5	34·8	31·2

changes in numbers. It may be inferred that (1) although the plates may be made with great care, the colonies of *Actinomyces* will not be evenly distributed if an unsuitable medium is used, and (2) the use of the standard medium in conjunction with proper methods of dilution and pouring is essential to obtaining consistent results.

It may be noted that the averages ($\bar{x}$) recorded in Table XVII were highly disproportionate to the corresponding figures in Table II. From the latter it may be gathered that the soil extract medium was so unfavourable to *Actinomyces* present in soils A, B, C and D that no more than one or two poorly formed colonies were observed on the plates. On the other hand, when used for plating the Chirakkal soil (Table XVII), the same medium yielded counts which, though inconsistent, were never below thirty per plate.

To determine whether the observation was due to the previous nutrition and condition of the cells (Subrahmanyan, *loc. cit.*), platings were made of soils A, B, C, D and the one from Chirakkal on mere tap-water set with 2·5 per cent. agar. It was observed that as in the case of soil extract agar practically no colonies appeared on plates made from A, B, C and D, while those from the Chirakkal specimen again averaged over thirty per plate. Since the tap-water could have contained only a small quantity of mineral matter, it may be inferred that the main nutrition of the cells was derived only from reserve materials which they had stored in the soil from which they came.

The foregoing observation and other similar ones referred to in the earlier part of the paper may be regarded as instances of a highly important aspect of microbial nutrition which has not so far received much attention and which, if fully studied, will help considerably in the proper understanding of the physiology of micro-organisms.

SUMMARY.

1. Most of the media commonly used for counting *Actinomyces* by plating were unsatisfactory. A starch medium containing calcium carbonate and minerals was found useful and offered prospects of being improved.

2. The clarity of the medium and the counts of *Actinomyces* were increased, and the undue growths of bacteria and fungi were suppressed, by effecting the following changes in the composition of the original medium:—(*a*) Reducing the quantities of starch, nitrogen and minerals to a minimum, (*b*) replacing calcium carbonate with calcium chloride, potato starch with soluble starch and ammonium sulphate

with a mixture of asparagine and sodium nitrate, and (*c*) maintaining the reaction at P_H 7·4. A medium composed of (in grams), soluble starch, 2·0; HK_2PO_4, 0·5; $MgSO_4$, $7H_2O$, 0·2; $CaCl_2$, 0·05; $Fe_2(SO_4)_3$, traces; $NaNO_3$, 0·05; asparagine, 0·05, in distilled water, 1000 c.c., with washed agar, 20–25 gms. was used. Final P_H 7·4 was developed and found to give satisfactory results.

3. Details relating to preparation of the medium, making of plates, temperature and period of incubation, and counting were standardised.

4. Studies on the consistency of the counts and distribution of colonies on the plates showed that the method developed was highly reliable.

The authors' thanks are due to Dr. R. V. Norris for his keen interest in the progress of the work and suggestive criticism.

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I.—BIOLOGICAL OXIDATION OF SULPHUR. PART II[1].
Effect on the Microflora of Activated Sludge.

By C. V. Ramaswami Ayyar and Roland V. Norris.

The value of microbiological analysis as a guide to the changes occurring in the soil has been much debated with a growing tendency to depreciate the reliability of inferences drawn from such work. The reason for this attitude lies in the scarcity of standard methods and the disregard of many factors limiting their applicability. Waksman (*Soil Sci.*, 1922, **14**, 343) has, however, shown that the plate method, when carried out under well defined conditions, gives much reliable information in regard to such changes, the number of the organisms thus revealed serving to indicate the soil conditions. Since the inferences drawn from such investigations have agreed with field results obtained with various crops over a number of years, it is clearly helpful to obtain information regarding the manner in which the soil population is affected by external conditions.

As in our previous investigation on the biological oxidation of sulphur (*J. Indian Inst. Sci.*, 1928, **11A**, 85), forming Part I[1] of the present series, we have used activated sludge, which is to be regarded as an intensified form of soil; the results may therefore be taken to indicate the general type of changes occurring in a soil population resulting from development of acidity. The work was also expected to indicate improved means for the isolation of sulphur-oxidising bacteria from this source.

EXPERIMENTAL.

Suspensions of activated sludge were aerated under different conditions for several weeks. Samples from each series were taken every 24 hours for enumeration and examination of microflora. Three sets of trials were conducted :—(1) Activated sludge alone, serving as control, (2) activated sludge with sulphur and (3) activated sludge with sulphur and mineral phosphate. The samples taken after suitable dilution (usually 1 in 40) were plated out on (1) nutrient agar, (2) wort-agar and (3) a special sulphur medium (*J. Bact.*, 1922, **7**, 606). The plates were incubated for 24 hours at 37° in the case of (1) and for 7 days at 30°, in those of (2) and (3). Comparative studies of the number and type of colonies developing on the plates were made. The reactions of the samples were also determined. The results are presented in Figures I, II and III.

Activated sludge alone.—There was very little change in reaction, the P_H remaining about 5·9 throughout. No marked decrease in bacterial numbers was observed as aeration proceeded. Considerable

changes, however, took place in the nature of the organisms found. At first spreading colonies predominated but soon died out, as did also putrefactive and chromogenic bacteria. It was however observed that the filamentous organisms believed to be responsible for the bulking of sewage (*J. Indian Inst. Sci.*, 1929, **12A**, 133) persisted in spite of continued aeration although the phenomenon has generally been regarded as a symptom of inadequate air-supply. The number of organisms developing on wort-agar or on the sulphur medium was small, the reaction not being favourable to the development of fungi or yeasts, and distinctly unfavourable to the growth of sulphur-oxidising organisms.

Activated sludge with sulphur.—In this series the results obtained were quite different. As aeration continued, owing to biological oxidation of sulphur, the acidity increased steadily, the reaction at the end of ten days being P_H 5·4. The conditions then seemed favourable to acid-producing organisms as there was an abrupt rise in acidity, the P_H after another week reaching 2·8. On continuing aeration, the rate of change slowed down considerably, but eventually a P_H of about 2·1 was attained after another 14 days' aeration.

This change in reaction had a marked influence on the flora. In the beginning, when the development of acidity was slow, the bacterial numbers as shown by agar counts tended to increase, this increase being particularly marked when the P_H reached 5·4. The agar counts in consequence reached a maximum between the 10th and 15th days. The development of further acidity, however, checked the increase of bacteria, and encouraged that of fungi and sulphur-oxidising organisms. The intermediate stage was characterised by a rapid increase of *Actinomyces* and moulds which became the predominating types, a result which is in close agreement with Waksman's observations in the case of soil (*Soil Sci.*, 1922, **14**, 74 and 151). At first the *Actinomyces* predominated, but as the acidity rose these decreased more rapidly than the moulds; among the latter, varieties of *aspergillus, fusarium, penicillium* and *trichoderma* were the most common. By the time, however, that the acidity reached a P_H of 2·4 most of the moulds also had died out with the exception of *fusarium* which seemed able to withstand a highly acid reaction, but did not survive when the P_H was about 2·2. No large increase in the number of sulphur-oxidising organisms took place until the P_H fell to 3·0. Counts on the sulphur medium then increased rapidly while those on nutrient agar and wort-agar became very low, the sulphur organisms not growing on those media. Practically all other types were now extinct. It was then easy to isolate sulphur-oxidising types even in liquid cultures, they being almost the sole survivors when oxidation was allowed to continue until a P_H of 2·2 was reached.

Fig. I

P_H Changes during the Oxidation of Sulphur

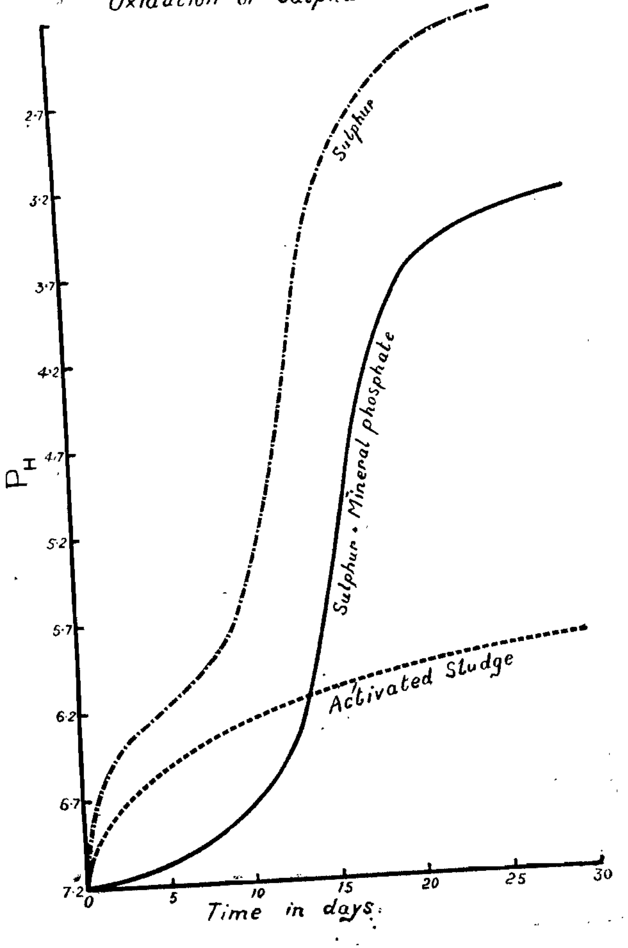
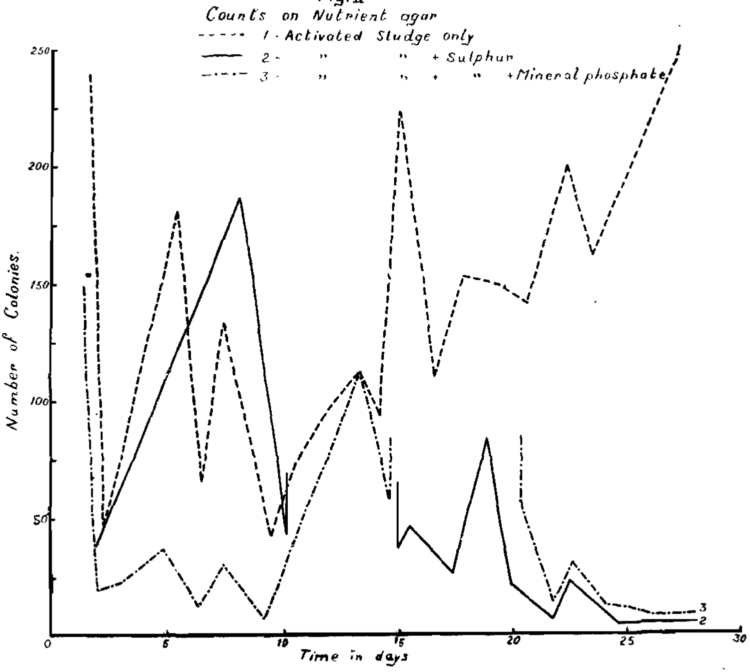


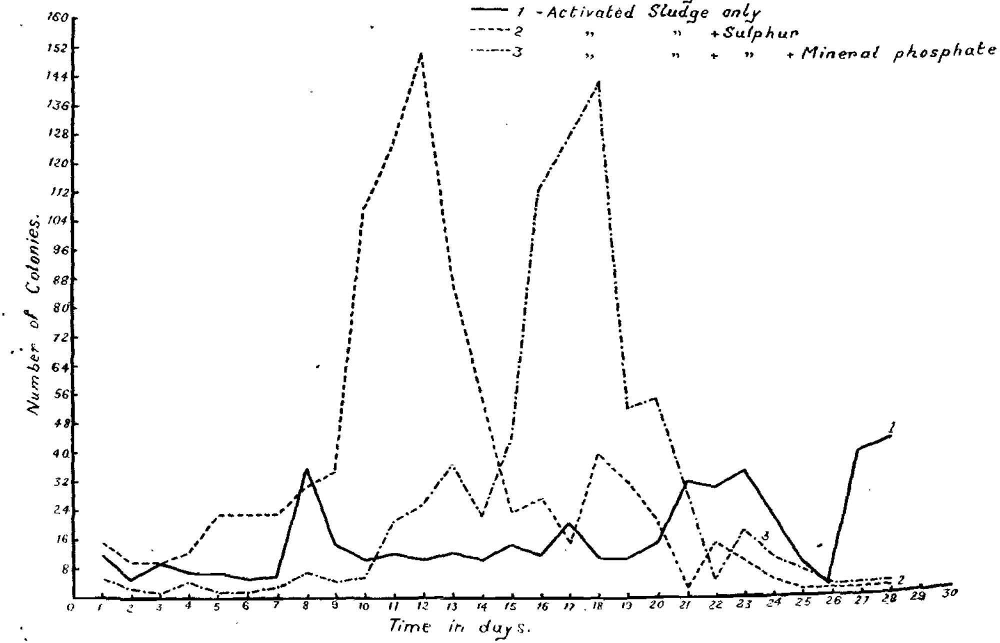
Fig. II



Counts on Nutrient agar
1 - Activated Sludge only
2 - " " + Sulphur
3 - " " + " + Mineral phosphate
Number of Colonies.
250
200
150
100
50
0
5
10
15
20
25
30
Time in days
3
2

Fig. III

Counts on Wort-Agar



Activated sludge, sulphur ana phosphate.—The results were intermediate in character between those found in series I and II. This was due chiefly to the buffer action of the phosphate added, the P_H in consequence undergoing less rapid variation. The phosphate added was a mineral phosphate from the Trichinopoly deposits and contained about 17 per cent. of calcium carbonate together with impurities such as iron and alumina. The initial P_H was slightly alkaline being about 7·2. Due to the buffer action already referred to (*J. Indian Inst. Sci.*, 1928, 11A, 87) the P_H changed but slowly in the initial stages and not till 10 days had elapsed did the figure fall to 6·6. The acidity then increased rapidly, the reaction changing from 6·6 to 4·0 in ten days, after which P_H 3·2 was reached.

In the beginning the bacterial counts were lower than in series I and II, but later increased, changes similar to those described under Series I taking place. Owing to the slower development of acidity, these were later in developing, increase in *Actinomyces* occurring between the 16th and 20th days. The counts on wort-agar and sulphur media varied somewhat from those previously described. The presence of lime from the mineral phosphate appeared to check the development of moulds and consequently the wort-agar counts did not show marked increases until the P_H had fallen to about 4·0. On the other hand, the multiplication of the sulphur-oxidising types took place at a rather earlier stage, the numbers beginning to rise when the P_H reached 3·2. It is possible the phosphate had a stimulating influence on those organisms.

SUMMARY.

During the progress of aeration and oxidation of sulphur there was decrease in the number of faecal and putrefactive bacteria and of chromogenic organisms. At a latter stage, further specialisation took place and only a few well defined soil types persisted. When the P_H reached about 5·2 *Actinomyces* were the predominating organisms, dying out at a reaction between 3 and 4, when moulds appeared in large numbers. The latter survived until the P_H was about 2·1, *fusarium* being usually the most resistant type. At this last stage, the sulphur-oxidising organisms began to multiply vigorously; they were almost the sole survivors and continued to increase and function actively. Owing to the absence of nearly all other organisms, it was then easy to isolate the sulphur-oxidising organisms in pure culture.

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II.—BIOLOGICAL OXIDATION OF SULPHUR. PART III.
A Sulphur-Oxidising Organism From Activated Sludge.

By C. V. Ramaswami Ayyar.

A review of recent literature on the isolation and study of organisms connected with the oxidation of sulphur and its compounds shows that the special organisms responsible for the process differ widely according to their source and the conditions of their normal activity. It has thus been shown that the reaction of media on which they are cultivated influences their behaviour to a marked extent, and that organisms isolated from sea-water, mud and sulphur-soil composts respectively exhibit specific differences in their morphological and physiological characters. These facts suggested that it might be possible to isolate from various other sources organisms which are able to bring about the oxidation of sulphur but differ in character from those previously described.

By composting sulphur, rock phosphate and soil it was found that the sulphur was rapidly oxidised to sulphuric acid which acted upon the tricalcium phosphate producing di- and monocalcium phosphates. In absence of a neutralising agent considerable quantities of acid accumulated in the compost, and by inoculating a suitable culture medium with such material, Waksman and Joffe (*J. Bact.*, 1922, **7**, 239) isolated a minute organism capable of rapidly oxidising elemental sulphur. Brown (*J. Amer. Soc. Agron.*, 1923, **15**, 350) succeeded in isolating a motile organism from activated sludge by inoculating a suitable liquid medium with fresh sludge, the organism being able rapidly to oxidise sulphur and produce acid. These observations of previous workers suggested the possibility of isolating a specific organism from suspensions of sulphur in activated sludge aerated until a high acidity developed.

ISOLATION OF THE ORGANISM.

In the foregoing paper it was shown that suspensions of sulphur in activated sludge, aerated for over a month, developed an acidity of P_H 2·1 and eliminated practically all the contaminating organisms, leaving the sulphur-oxidisers as almost the sole survivors. If the

mixture is then directly inoculated into the sterilised liquid medium of Waksman and Joffe (*loc. cit.*) and incubated at 28–30°, the growing organisms render the culture medium uniformly cloudy within a week when sulphur is present as the chief source of energy. By repeated sub-culturing in the liquid medium pure cultures of sulphur-oxidising organisms were obtained. In presence of a neutralising agent such as tricalcium phosphate, there was a characteristic formation of gypsum crystals which appeared concurrently with the development of cloudiness. The P_H of the medium remained stationary at about 2·4 with the conversion of the tricalcium into monocalcium phosphate.

EXPERIMENTAL.

The methods and media employed were mainly those of Waksman and Joffe (*J. Bact.*, 1922, **7**, 606) with certain modifications. The liquid medium contained (in grams per 1000 c.c. of distilled water):—$(NH_4)_2SO_4$, 0·2; $MgSO_4$, 0·1; $FeSO_4$, 0·01; H_2KPO_4, 5·0; $CaCl_2$, 0·25; Sulphur, 10·0. The solutions were prepared in bulk and measured in 500 c.c. portions into 850 c.c. conical flasks plugged with cotton wool and sterilised by steaming for 30 minutes on three consecutive days. The inoculations were made from a seven-day old culture and the flasks incubated at 28–30°; the P_H of the medium was 4·6.

Washed agar medium.—The agar was prepared by washing the fibres in distilled water for several days and drying at 60° (*J. Bact.*, 1920, **5**, 591); a 2·5 per cent. solution was filtered clear, tubed in 10 c.c. portions and sterilised in an autoclave at 15 lbs. The following solutions were prepared (in grams per 100 c.c.) and sterilised:—(1) H_2KPO_4, 3·0; (2) NH_4Cl, 0·1; $MgCl_2$, 0·1 and $CaCl_2$, 0·25; (3) $Na_2S_2O_3 \cdot 5H_2O$, 5·0.

The agar was melted, cooled to 40° and plates made by placing 1 c.c. each of solutions 1, 2 and 3 in sterile petri-dishes, adding the desired inoculum and then the melted agar. The solutions were ten times the usual strength and were used in the preparation of agar or silica gel media.

Silicic acid gel.—The method of Beijerinck as described by Gibbs (*Soil Sci.*, 1919, **8**, 439) combined with the modification suggested by Waksman and Carey (*J. Bact.*, 1926, **12**, 90) gave satisfactory results. From a normal solution of hydrochloric acid, 5 c.c. portions were placed in a series of test tubes and varying amounts of an equivalent solution of sodium silicate added. The contents of the tubes were immediately mixed and poured into dishes, and the mixture where the gel formation took place within two to five minutes was chosen. It was found that

5 c.c. of silicate solution gave the best results. To 15 c.c. of normal hydrochloric acid 15 c.c. of 8 per cent. silicate solution was added and the mixture immediately shaken and poured into petri-dishes which rested on a level surface until the gel was well formed. They were then placed in wash-basins, dialysed in running tap-water until free from chlorides and then transferred to sterile vessels containing boiled distilled water, this being replaced several times. After draining, they were flamed to sterilise the surface of the gel, on to which was poured the sterile concentrated nutrient solution ; this was allowed to diffuse through the gel for about 10 minutes, the excess was poured off and the surface of the gel once again flamed to ensure sterility. The medium was then inoculated with a drop of the culture spread evenly over the plate which was then allowed to remain in the incubator at 28–30°. The character of the growth was very satisfactory for the study of colonies.

Morphology.—The organism when grown on the synthetic medium described above, consisted of short rods with rounded ends, usually occurring in pairs. Spore formation was absent. The cells were motile in the earlier stages and remained so during over a month. By adopting the new method of gram-staining (Burke, *J. Bact.*, 1922, **7**, 178) the organism was found to be gram negative. Deposition of sulphur did not occur within or without the cells (Figure I).

In the course of the morphological study of the organism the presence of long filamentous forms were occasionally observed. At first they were believed to be due to contamination, but a careful study of hanging drop preparations showed that they were only involution forms of the sulphur-oxidising organism, appearing in old cultures or when conditions became unfavourable. The involution forms varied in length sometimes reaching 100 μ with numerous forms intermediate in size (Figure II). They were non-motile and differed in physiological activity from the normal, developing most quickly when the temperature was too high for normal growth, i.e., between 35° and 37° ; if the temperature is again lowered, they disappear and the organism regains its normal characteristics.

Agar plates.—Plates of washed agar were prepared as previously described, inoculated from one of the pure cultures on liquid medium and incubated at 28–30°. In seven days, colonies began to appear; they were small, but visible to the naked eye as an opaque, creamy colony. Examination under the microscope revealed a central nucleus with a creamy, uniform margin (Figure III).

Silicic acid gel plates.—The colonies obtained by using silicic acid gel were more satisfactory than those on washed agar, which they

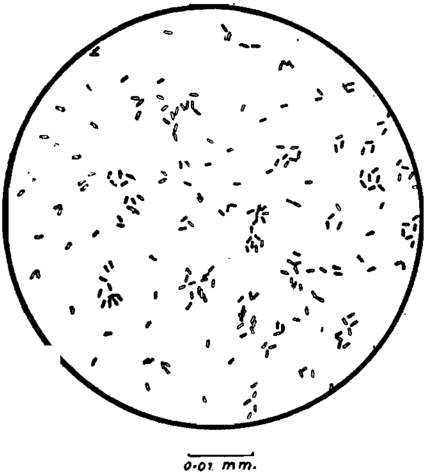


Fig. I
Normal Forms

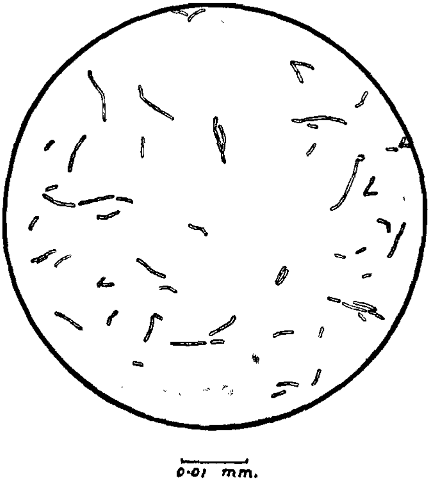


Fig. II
Involution - Forms.

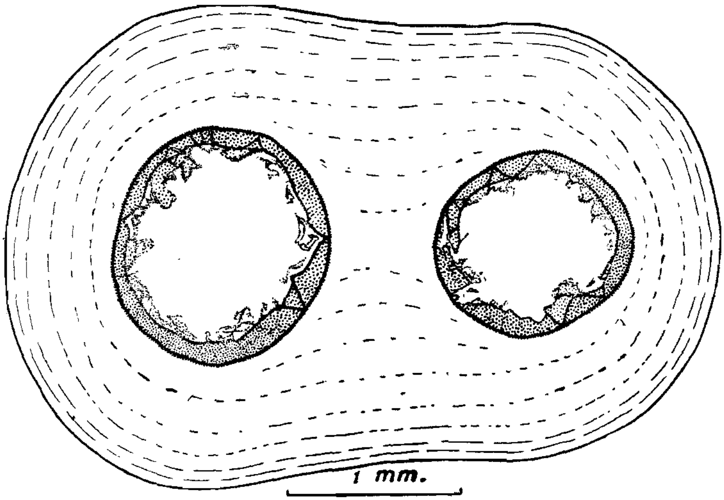
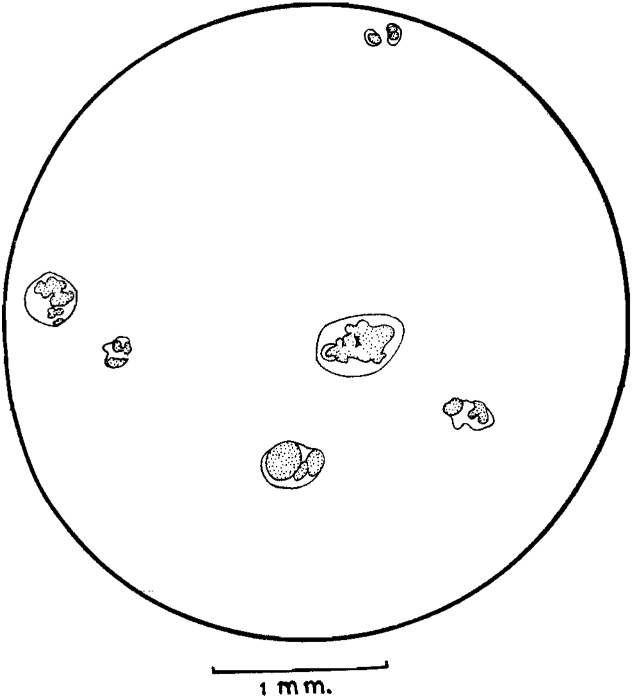


Fig. III
Colonies on agar.



Colonies on Silica Jelly

Fig. IV

resembled but were somewhat more dense. They began to appear in two days and developed rapidly all over the plate attaining their maximum development in 7 to 10 days, increasing in density from the margin to the centre, the former being wavy in appearance under the microscope. The organisms exhibited the same characteristics as those grown on liquid medium, but appeared slightly protuberant due to the change in the environment (Figure IV).

Gypsum blocks.—These were prepared as usual, and having been sterilised by heating in an electric muffle furnace at a temperature of 400° for half an hour, were transferred to petri-dishes containing 50 c.c. of a liquid in which thiosulphate was the source of sulphur. They were inoculated on the surface from a pure culture grown in liquid medium and incubated at 28–30°. Pale brown colonies developed, in size equal to those on agar plates. By repeated sub-culturing in liquid medium the organism could be kept alive and active for any length of time. It was often noticed that there was a decided increase in their activity.

Physiology.—Besides deriving all its carbon from the carbon dioxide of the atmosphere, the organism was able also to utilise carbohydrates and glycerol, but not carbonates which even in small amounts were toxic because in their presence the medium remained alkaline throughout, a condition unfavourable to the growth of the organism. Sulphur and thiosulphate were the important sources of energy to the organism. The amount of sulphur oxidised and acid produced were greater in presence of carbohydrates than in their absence, indicating the beneficial influence of such compounds. The organism grew equally well whether the medium contained elementary sulphur or thiosulphate, the cloudiness appearing in either case within a week's time. Sulphides and sulphites were not utilised. Mere traces of inorganic salts in addition to phosphates were sufficient for growth. In absence of nitrogen the organism did not normally thrive and the presence of ammonium salts such as the sulphate assisted its growth. In presence of mannitol, however, the organism seemed able to make good growth even in absence of ammonium salts, suggesting that it might be able to assimilate atmospheric nitrogen. This aspect of the question is being further investigated.

Influence of various inorganic and organic compounds on the amount of sulphur oxidised.—A detailed study has been made of the influence of various inorganic and organic compounds on the oxidation of sulphur by a pure culture of the organism. The medium was distributed in 500 c.c. portions in 850 c.c. flasks containing 5 gms. of sulphur flowers and 0·1 per cent. of salt under test, the flasks being sterilised in steam in the usual way. The organic compounds were

sterilised separately and then added to the sterile medium. The flasks were all inoculated with 1 c.c. of a 1-in-50 dilution of a 7-day old culture of the organism and incubated at 28–30°, 50 c.c. portions being withdrawn from time to time with sterile pipettes for analysis. The P_H was determined by the colorimetric method using the colour standards of Medalia (*J. Bact.*, 1920, **7**, 589) and the total acidity estimated by titrating 5 c.c. of the culture medium against N/50 sodium hydroxide using phenolphthalein as indicator. The water-soluble sulphates were determined gravimetrically.

The amounts of sulphur oxidised under various treatments as compared with sulphur alone are shown in Figures V and VI. The amount of sulphur oxidised per 100 c. c. in the control was 65 mgms. in 40 days, whereas in the presence of ammonium sulphate, aluminium sulphate and sodium silicate over the same period the amounts were 438·0, 307·0, and 278·0 mgms. respectively. In presence of organic compounds such as lactose, mannitol and glycerol the amounts oxidised were 256·0, 223·0 and 185·2 mgms. respectively. From this it will be observed that while such organic compounds had beneficial influence on sulphur-oxidation they were less effective than inorganic salts.

The general behaviour of the cultures under these different treatments requires some explanation. Although there was considerable difference in the initial reaction of all the various media, the fall in P_H was quite rapid in all and reached 1·2 within 40 days, there being a progressive development of titratable acidity in all the cultures. The behaviour of sodium silicate was the most remarkable. Initial P_H was 6·6 and the organism did not show any activity for over 10 days being able, presumably, to acclimatise itself to the new conditions; it eventually began to grow rapidly, P_H being reduced to the level of other cultures in 30 days, and the amount of sulphur oxidised comparing favourably with the rest of the series. This indicates that in the silicate medium the P_H limits are from 6·6 to 1·4, far higher than those observed by Joffe (*loc. cit.*) for his organism. With aluminium sulphate, manganous sulphate, ammonium sulphate and tricalcium phosphate, the P_H was favourable from the beginning, and the organisms increased rapidly and oxidised more sulphur. The effect of ferrous sulphate was not markedly favourable and was inferior to that of the other salts studied. Throughout the series accumulation of free acid rose proportionately with the amounts of sulphur oxidised as indicated by the titration figures shown in Tables I and II. The P_H values of the series are shown in Tables III and IV.

In presence of organic compounds it was observed that the series uniformly started with P_H 5·0, the fall therefore measuring the influence

Fig. V

Influence of inorganic salts

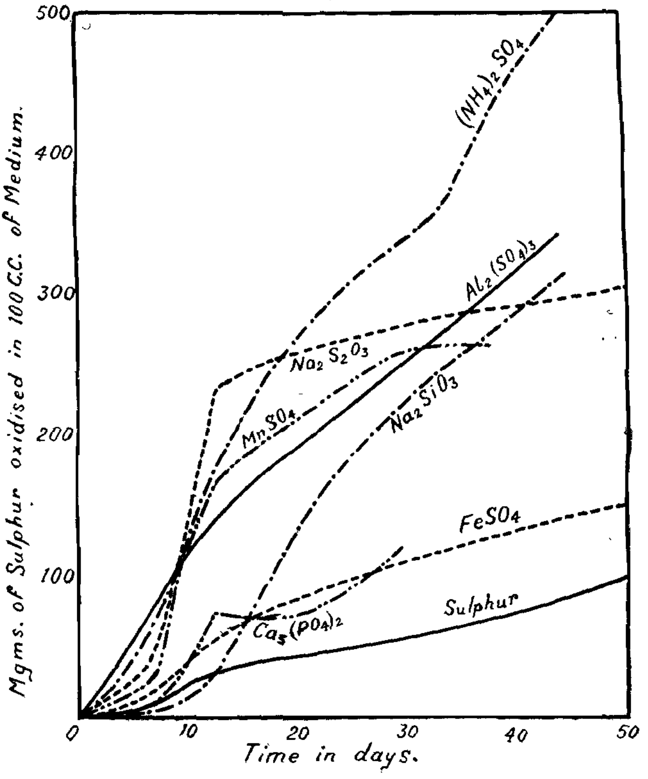


Fig. VI
Influence of organic Compounds.

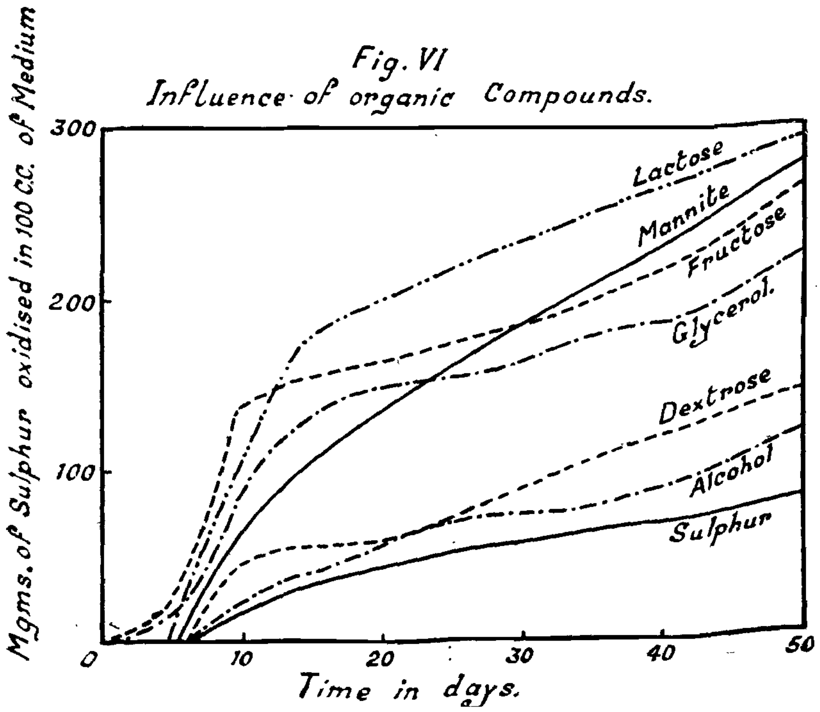


TABLE I.

INORGANIC SALTS SERIES.

Titratable Acidity in c.c. of N/50 Sodium Hydroxide for 5 c.c. of Medium.

—	Time in days						
	Start	7	12	20	30	40	50
Sulphur only	5·5	5·7	9·6	12·3	15·9	...	22·1
Sodium thiosulphate only ...	5·4	5·5	20·3	22·2	27·5	...	30·8
With aluminium sulphate ...	6·4	17·5	28·2	34·5	45·1	54·1	60·3
,, manganous sulphate ...	6·5	12·6	31·5	39·9	45·5	...	...
,, ammonium sulphate ...	6·7	14·4	32·2	41·9	57·5	72·5	83·4
,, ferrous sulphate ...	6·1	8·9	14·0	18·1	22·0	26·1	31·3
,, calcium phosphate ...	5·5	7·0	16·8	22·7	24·3	25·4	26·4
,, sodium silicate ...	3·5	3·5	6·1	20·8	32·5	44·3	51·1

TABLE II.

ORGANIC COMPOUNDS SERIES.

Titratable Acidity in c.c. of N/50 Sodium Hydroxide for 5 c.c. of Medium.

—	Time in days						
	Start	7	12	20	32	40	50
Sulphur only	5·5	5·7	9·6	12·3	15·9	...	22·1
With dextrose	5·4	5·3	16·5	18·1	20·2	22·4	26·9
,, glycerol	5·4	5·7	17·4	27·3	30·4	33·4	40·2
,, alcohol	5·3	5·9	10·5	14·2	16·9	19·0	23·4
,, mannitol	5·2	5·3	14·5	23·7	34·8	39·0	49·4
,, lactose	5·3	5·7	17·2	33·7	40·6	44·3	51·0
,, fructose	5·3	7·9	23·7	29·3	32·8	37·1	47·0

TABLE III.

Inorganic Salt Series ; Change in P_H.

—	Time in days						
	Start	7	12	20	30	40	50
Sulphur only	5·1	3·8	2·4	2·2	1·9	1·7	1·7
Sodium thiosulphate	5·0	4·6	1·8	1·8	1·8	1·7	1·7
Sulphur + aluminium sulphate	2·8	1·9	1·7	1·5	1·2	1·2	1·2
,, + manganous sulphate	4·5	2·2	1·5	1·4	1·3	1·3	1·2
,, + ammonium sulphate	4·7	2·3	1·6	1·3	1·2	1·2	1·2
,, + ferrous sulphate	3·2	2·5	2·0	1·8	1·7	1·7	1·7
,, + calcium phosphate	4·9	3·0	2·8	2·5	2·4	2·4	2·4
,, + sodium silicate	6·6	6·6	3·5	1·7	1·6	1·3	1·3

TABLE IV.

Organic Compounds Series ; Change in P_H.

—	Time in days						
	Start	7	12	20	30	40	50
Sulphur only	5·0	3·8	2·4	2·2	1·9	1·7	1·7
With dextrose	5·0	3·3	1·9	1·9	1·7	1·7	1·7
,, glycerol	5·0	4·4	1·8	1·7	1·6	1·6	1·3
,, alcohol	5·0	3.4	2·3	2·1	1·9	1·8	1·7
,, mannitol	5·0	3·8	1·9	1·7	1·6	1·4	1·2
,, lactose	5·0	3·7	1·8	1·6	1·4	1·3	1·2
,, fructose	5·0	2·6	1·7	1·6	1·6	1·6	1·4

of the substances added. All the sugars disappeared rapidly, lactose and mannitol seeming to be the carbohydrates with maximum stimulating action, followed by glycerol and fructose. Joffe found dextrose to be more favourable for his cultures. Gas was not evolved, but the

characteristic cloudiness observed in the medium was even more marked in these cultures. Sulphur-oxidation was not as vigorous as in the inorganic media.

The stimulating action of such inorganic salts on biological processes has been noticed previously, and their influence on nitrification and ammonification studied by Brown and Munges (*Soil Sci.*, 1916, **3**, 67) and others. It has been argued that the salts of aluminium undergo hydrolysis producing a strongly acid medium. With initial P_H 2·8 it would be expected that the sulphur-oxidising organisms, being able to tolerate such high acidity, would thrive ; while others, less adaptable organisms such as nitrifiers and ammonifiers, would not. Consequently it was not surprising to find that aluminium salt showed a distinctly stimulating effect. On the other hand, the superiority of ammonium sulphate to aluminium sulphate could be traced to the favourable effect of easily available nitrogen, readily utilised by the organisms for their development and activity. In the case of sodium silicate, however, the remarkable effects are most probably attributable to the increased surface presented by the colloidal silicic acid, and not to the reaction of the medium which approached neutrality.

Influence of organic compounds.—It has been mentioned above that carbohydrates were favourable to the bacterial oxidation of sulphur; this was observed also by Waksman and Joffe (*loc. cit.*) who attributed the stimulation to their utilisation by the organisms. Starkey (*J. Bact.*, 1925, **10**, 192) found that dextrose disappeared from the media with growth of the organisms during incubation and a definite correlation existed between its disappearance and the amount of acid produced. Hence he also attributed the stimulation to the assimilation of carbon from dextrose.

It is therefore desirable to ascertain whether the easily available carbon compounds would serve as a source of carbon for the organisms in absence of atmospheric carbon dioxide. Sterile 100 c.c. portions of the synthetic medium in 250 c.c. Erlenmeyer flasks, containing the various organic compounds in the same concentration as before, were inoculated with a pure culture of the organism and placed under bell-jars covered with black paper and sealed with tap-grease. The jars containing the cultures were aspirated with air passed through 100 per cent. caustic potash and soda-lime tubes. Similar guard-tubes were also provided on the other side of the bell-jars close to the pump by which air was drawn into the system. Care was taken to renew the solution as frequently as possible during aeration ; a corresponding series was run under normal conditions over the same period. The cultures were analysed after one month.

In comparing the activity of the respective series, the amount of sulphur oxidised in the sulphur series without the organic compound was taken as unity ; the ratios are given in Table V.

TABLE V.

Substance added	Ratios of sulphur oxidised	
	Ordinary air	Air free from CO_2
Sulphur only ...	1·00	1·00
,, + glucose ...	1·60	0·56
,, + lactose ...	2·95	2·27
,, + mannitol ...	4·42	3·06
,, + glycerol ...	3·42	1·75

It will be seen that in absence of carbon dioxide the organisms did not utilise glucose, while the other organic compounds stimulated the oxidation of sulphur, but not to such an extent as under normal conditions. The figures support the view that this activation has been effected by the assimilation of readily available carbon from the organic compounds studied. Under normal conditions the acceleration was proportionately greater as the carbon could be obtained from the air and from the organic compounds. This observation confirmed the view expressed by Starkey (*loc. cit.*) that in the presence of organic compounds a general stimulation is noticeable, due to utilisation of carbon from the substances supplied.

Influence of aeration.—Ranganathan and Norris (*J. Indian Inst. Sci.*, 1927, **10A**, 114) have observed that the supply of air to a culture containing ammonium salts in presence of nitrifying organisms hastens the nitrification process leading to the accumulation of nitrates. Hence it was thought that a continuous supply of air to a culture of sulphur-oxidising organisms would accelerate oxidation of sulphur. A culture of the organism incubated at 28–30° was aerated, and periodical samples withdrawn under sterile conditions to determine titratable acidity, P_H and sulphur oxidised. Similar determinations were conducted with an unaerated sample. The results are recorded in Table VI, indicating a general fall by 50 per cent. in the amount of sulphur oxidised in the aerated culture as compared with the unaerated. Even though there had been a slight fall in P_H with rise in titratable acidity, these did not compare favourably with the unaerated series, confirming the observation that additional air disturbing the

culture adversely affects the normal growth and development of the organisms, with considerable diminution in their activity.

TABLE VI.

Rate of sulphur oxidised with and without aeration.

Analysis after	Mgms. of sulphur in 100 c.c.		Diminution in sulphur oxidised, mgms.	P_H		Titratable acidity in c.c. N/50 NaOH per c.c. medium	
	Aerated	Un-aerated		Aerated	Un-aerated	Aerated	Un-aerated
Control ...	8·78	8·78	...	4·8	4·8	1·10	1·10
7 days ...	9·32	9·90	0·58	3·8	3·8	1·16	1·24
12 ,, ...	19·23	32·40	13·17	2·7	2·4	1·46	1·92
20 ,, ...	31·59	50·50	18·91	2·4	2·2	1·79	2·46
30 ,, ...	41·20	70·80	29·60	2·3	1·9	2·08	3·20

Influence of Light.—The effect of exposing micro-organisms to direct sunlight, diffused light, polarised light and in darkness has been recorded (Giltner, *Microbiology*), it being found that direct sunlight is for the most part fatal to their growth. Whilst diffused light exerts a beneficial influence on some fungi, most organisms develop well in darkness and function normally. From a study of the influence of diffused light and of darkness on the amount of sulphur oxidised, it was found that after an incubation period of 45 days the amounts of sulphur oxidised were 174 and 368 mgms. respectively in 100 c.c. of medium. The results indicated that, although diffused light did not completely retard growth and activity, darkness was more favourable to them. This observation agrees with those recorded with many soil organisms preferring darkness to light, and as the sulphur-oxidisers are commonly present in all soils, their behaviour appears quite normal.

Influence of Charcoals.—The important effect of surface phenomena in biological reactions has been well recognised, and chemical changes produced by organisms mostly occur at surfaces. The relative acceleration in activity has also been shown to depend on the dispersion of the substrate, finer division leading to greater activity. In sulphur-oxidation it was found by Starkey (*J. Bact.*, 1925, **10**, 154) that different forms of sulphur varying only in their fineness of distribution in the liquid phase affected markedly the rate of oxidation. In the case under investigation, it was found that in any hanging drop culture

examined under a microscope with a few particles of sulphur, the organisms clung to the surface of the particles which gradually disappeared in the course of a few minutes, suggesting that the reaction occurred at the surface of contact of the organisms and the substrate; hence the finer it is, the more rapid will be the conversion of sulphur to sulphuric acid. Any process enlarging the surface exposed should lead to increased oxidation by the organisms.

It has been known that charcoals are definite catalysts to oxidation, possessing enormous surface and high power of adsorption; hence it was thought that their introduction into cultures might lead to an increased rate of oxidation. Moreover, an interesting observation has been recorded by Hutchinson (*Sci. Rep. Agri. Res. Inst. Pusa*, 1924–25, 38) that addition of charcoal in small quantities to a sulphur-phosphate compost enhanced the rate of phosphate solubilisation. No reason has been assigned to the acceleration, but it is quite probable that this might have resulted in the increased oxidation of sulphur with consequent production of sulphuric acid, this in turn hastening the production of soluble phosphate. This solitary and remarkable observation suggested a detailed inquiry into the general behaviour of charcoals, both vegetable and animal, to explain the mechanism of acceleration.

At this stage, it is enough to investigate whether there was any acceleration in oxidation of sulphur due to the introduction of various charcoals in the least quantity possible as in the experiments cited above. In the experiments to be reported hereunder only 0·05 gm. of the respective dry charcoals has been employed. To 100 c.c. of liquid medium contained in 250 c.c. conical flasks was added 0·05 gm. of the charcoals, sterilisation being effected by flowing steam for 30 minutes in 3 days. Inoculation with one c.c. of the pure culture followed and incubation at 28–30°, periodical analysis being made for titratable acidity, P_H and amount of sulphur oxidised. These are given in Tables VII, VIII and IX. It will be seen from the tables that there has been an all round acceleration in the amount of sulphur oxidised with all the charcoals irrespective of their source, the stimulating influence being at a maximum in the norit series. The nature and mechanism of this activation will be discussed in a later paper. The ratio of increased oxidation varied from 1·5 to 3·0 when compared with the sulphur series without charcoal. With the fall in P_H there was a corresponding increase in titratable acidity, which might be due to various factors. These may be the increase in the amount of surface introduced by the charcoals, their electro-kinetic behaviour, or the displacement of bases from the charcoals liberating some stimulating bases like aluminium or manganese, which have been previously shown to accelerate the oxidation of sulphur. Alternatives are general

stimulation of growth or adsorption of gases from the air which might be readily utilised by the organisms for their cell respiration and development with the consequent increase in activity. Other physical factors such as surface tension and viscosity, which numerous workers have shown to be effective in modifying profoundly the behaviour of micro-organisms, might also account for the observed stimulation. These possibilities are being investigated.

TABLE VII.

Amount of sulphur oxidised in mgms. per 100 c.c. of medium.

No.	—	Time in days					
		Start	10	20	30	40	50
1	Control	6·04	6·04	7·42	7·69	8·24	8·24
2	Inoculated	7·69	29·12	56·31	66·92	77·18	87·35
3	,, + Animal charcoal ...	7·69	10·16	35·98	79·38	84·87	97·24
4	,, + Norit	7·69	63·46	104·65	130·47	159·90	209·6
5	,, + Coconut charcoal..	6·59	32·14	53·02	90·93	102·18	121·7
6	,, + Wood charcoal ...	7·14	12·64	50·82	89·08	104·11	172·8
7	,, + Polycarbon ...	7·14	10·16	65·67	83·78	103·9	139·0

TABLE VIII.

Titratable acidity in c.c. of N/50 Sodium Hydroxide per c.c. of medium.

No.	—	Time in Days					
		Start	10	20	30	40	50
1	Control	1·88	1·88	1·88	1·88	1·88	1·88
2	Inoculated	1·88	2·48	3·33	3·76	4·08	4·46
3	,, + Animal charcoal ...	1·80	1·88	2·63	4·04	4·20	4·62
4	,, + Norit	1·84	3·51	4·78	5·64	6·24	8·03
5	,, + Coconut charcoal ...	1·84	2·54	3·16	4·22	4·83	5·34
6	,, + Wood charcoal ...	1·80	2·01	3·10	4·20	4·61	6·92
7	,, + Polycarbon ...	1·84	1·88	3·57	4·39	4·62	5·96

TABLE IX.

Change in P_H.

No.	—	Time in Days					
		Start	10	20	30	40	50
1	Control	4·6	4·6	4·6	4·6	4·6	4·6
2	Inoculated	3·8	3·0	2·8	2·6	2·6	2·2
3	,, + Animal charcoal ...	4·4	4·4	3·2	2·8	2·6	2·0
4	,, + Norit	4·2	2·4	2·0	1·6	1·4	1·4
5	,, + Coconut charcoal ...	4·2	3·0	2·8	2·4	2·4	1·8
6	,, + Wood charcoal ...	4·4	4·2	2·8	2·4	2·4	1·6
7	,, + Polycarbon ...	4·3	4·2	2·5	2·2	2·2	1·6

Sulphur-Carbon ratio of the sulphur-oxidising organism.—The general morphological characters of an organism do not satisfactorily settle the question of its identity, for most of the observations are purely qualitative; but they may be used with advantage provided definite quantitative data are revealed by its physiological behaviour. In the study of similar autotrophic oxidising organisms it has been found that a correlation existed between the amounts of carbon assimilated and of substrate oxidised. This relationship has been ascertained in the case of nitrifying organisms by Meyerhof (*Arch. ges. Physiol.*, *1916*, **164**, *353*) and of the sulphur-oxidising organism isolated from soil-sulphur compost by Waksman and Starkey (*J. Gen. Physiol.*, 1923, **5**, 289). Thus the sulphur-carbon ratio of the organism isolated from activated sludge should decide whether this is identical with the one so well studied by the foregoing authors; accordingly, the question has been studied with the normal forms of organisms at 28° and with the involution forms at 35° to ascertain definitely the variations due to alteration in the structure of the organisms.

The usual liquid medium of Waksman and Joffe was employed, but conductivity water was used in its preparation. The sulphur was weighed into the individual flasks of 850 c.c. capacity which had been cleaned previously with potassium dichromate-sulphuric acid mixture. The medium (300 c.c.) was placed in the respective flasks, plugged with cotton wool and sterilised in flowing steam for 30 minutes on three consecutive days, all manipulations being conducted under strictly sterile conditions; one c.c. from a seven-day culture of actively growing organism was used for inoculating each flask. Some flasks were not inoculated and thus served as controls.

Methods of analysis.—Acidity in 5 c.c. of the filtered medium was titrated with N/10 sodium hydroxide using phenolphthalein as indicator. The hydrogen-ion concentration was determined colorimetrically using the colour standards of Medalia (*loc. cit.*) with the indicator series of Clark and Lubs. Sulphate was estimated gravimetrically as barium sulphate. Total solids were determined by evaporating the filtered culture in the desiccator over sulphuric acid in vacuum and weighing the residue. The total carbon of the culture was estimated by means of the wet combustion apparatus resembling that of Waksman and Starkey (*J. Gen. Physiol.*, 1923, **5**, 289), the details of estimation being strictly observed. The accuracy of the method was checked with pure sucrose which gave nearly 98 per cent. of the theoretical yield. Carbon was estimated in the control and inoculated samples at the end of various incubation periods, using 100 c.c. of unfiltered medium; the balance was filtered and used for other determinations. The results are tabulated in Tables X and XI.

In presence of the normal forms, sulphur-oxidation and carbon-assimilation progressed rapidly; with increase in the carbon assimilated there was a corresponding rise in the amount of sulphur oxidised. In the course of 45 days 36 per cent. of the sulphur was oxidised with a fall in the hydrogen-ion concentration to less than P_H 1·4. The ratio of the sulphur oxidised to carbon assimilated varied from 22·4 at the beginning of active growth to 46·95, falling to 40·85 at the end of the experiment, with an average of 40·75 parts of sulphur oxidised for every gram of carbon assimilated as carbon dioxide from the atmosphere. During the early stages the ratio was small indicating the economic utilisation of energy at the active growth phase, but with multiplication of organisms there was an increased intake of carbon with a proportionate rise in the sulphur oxidised, the sulphur-carbon ratio of the organism isolated by Joffe being 31·8 (*loc. cit.*). As the organisms developed under normal conditions of temperature and medium composition, the sulphur-carbon ratio of 40·75 seems to be quite characteristic of the activated sludge organism; but it has been observed by Starkey (*J. Bact.*, 1925, **10**, 160) that with initial concentrations of 1 and 5 per cent. sulphuric acid the ratios were 36·6 and 43·0 respectively, with the organisms having 31·8 as the ratio. Hence under favourable conditions the organisms might have a high sulphur-carbon ratio. Such conditions being absent with the observations herein recorded, we are justified in classifying this as a distinct species of sulphur-oxidising organism.

In presence of involution forms working at a temperature (35°) most suited to their development, the amount of carbon assimilated was very small, and the sulphur oxidised was also low. If the same

TABLE X.

The Sulphur-Carbon Ratio with Normal forms.

Days	Mgms. of Carbon in 100 c.c. of medium.			Mgms. of carbon assimilated	Mgms. of sulphur as SO_4 in 100 c.c. of medium.			Mgms. of sulphur oxidised	Ratio of Sulphur to Carbon	P_H	Titer N/10 NaOH per c.c. of medium.	Total solids: mgms. in 100 c.c.
	1	2	Average		1	2	Average					
0 Control	2·59	1·78	2·18	...	7·69	8·24	7·96	...	...	4·6	0·39	570
26 ,, ...	2·46	2·04	2·25	...	7·69	8·24	7·96	...	...	4·6	0·38	580
42 ,, ...	2·49	2·01	2·25	...	7·69	7·69	7·69	...	...	4·6	0·38	570
Average ...			2·23				7·87					
6 Inoculated	4·24	3·71	3·98	1·75	46·70	47·25	46·98	39·11	22·35	3·0	0·63	710
12 ,, ...	4·31	4·31	4·31	2·08	100·60	100·60	100·60	92·73	44·63	2·4	0·96	890
16 ,, ...	6·12	5·30	5·71	3·48	143·38	145·03	144·21	136·34	39·13	1·8	1·30	1,080
23 ,, ...	7·80	5·52	6·66	4·43	212·04	212·04	212·04	204·17	46·07	1·6	1·65	1,520
30 ,, ...	7·44	6·72	7·08	4·85	236·77	234·58	235·68	227·81	46·95	1·6	1·79	1,600
37 ,, ...	8·55	8·76	8·66	6·43	270·30	270·30	270·30	262·43	40·83	1·4	2·04	1,760
45 ,, ...	10·08	10·38	10·23	8·00	370·26	370·26	370·26	362·39	45·29	1·4	2·63	2,310
								Average	40·77			

TABLE XI.

The Sulphur-Carbon Ratio with Involution forms.

Days	Mgms. of Carbon in 100 c.c. of medium			Mgms. of carbon assimilated	Mgms. of sulphur as SO_4 in 100 c.c. medium			Mgms. of sulphur oxidised	Ratio of Sulphur to Carbon	P_H	Titer N/10 NaOH per c.c. of medium	Total solids, mgms. in 100 c.c.
	1	2	Average		1	2	Average					
0 Control	1·65	1·96	1·80	...	8·79	8·36	8·57	...	...	5·0	0·22	360
20 ,, ...	2·50	2·18	2·34	...	8·79	9·18	8 98	...	...	5·0	0·23	366
Average ..	...	...	2·07	...	...	...	8·77	...	...	...	...	...
5 Inoculated	2·28	2·46	2·37	0·30	22·41	21·32	21·86	13·11	43·92	3·4	0·31	400
9 ,, ...	2·75	2·71	2·73	0·66	37·14	37·36	37·25	28·49	43·35	3·0	0 40	440
16 ,, ...	3·34	3·34	3·34	1·27	52·74	52·52	52·63	43·88	34·68	2·5	0·50	526
30 ,, ...	3·37	3·65	3·51	1·43	58·23	59·88	59·06	50·30	35·12	2·3	0·55	560
40 ,, ...	4·52	4·99	4·76	2·69	82·40	83·50	82·95	74·20	27·63	2·0	0·72	672

volume-weight of the organisms both of normal and involution forms is considered from the point of view of the total surface, it will be evident that the involution forms would naturally possess much less surface than the normal ones. As the rate of the reaction is proportional to the reacting surface, the energy requirements of the involution forms will be proportionately much less than those of the normal ones. Hence it is but natural to expect less intake of carbon by the involution forms with a corresponding decrease in the amount of sulphur oxidised. The velocity of oxidation in the latter instance has been reduced by 33·8 per cent. during the period of observation.

SUMMARY.

The cultural and physiological characteristics of a sulphur-oxidising organism isolated from activated sludge are described.

The organism develops on washed agar, silica jelly and gypsum blocks presenting both normal and involution forms; the characteristics and cultural behaviour are different from those observed by Joffe and co-workers. It withstands P_H 6·6 and can utilise carbohydrates to some extent, oxidising more sulphur in their presence. Certain inorganic salts also have a favourable effect, the influence of sodium silicate being very marked.

Additional air resulting in disturbance of the culture retards the progress of sulphur-oxidation.

The influence of vegetable and animal charcoals has been studied and is found to accelerate oxidation.

The sulphur-carbon ratio of the organism is 40·75; that of Joffe's 31·8. Thus the organism is much more economical in the utilisation of energy.

Peculiarities in the morphological and physiological behaviour, the higher sulphur-carbon ratio and other similar considerations afford data sufficient to classify the organism as a new species, capable of oxidising elemental sulphur to sulphuric acid and not accumulating sulphur within or without the cells.

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CONTRIBUTIONS TO THE STUDY OF SPIKE-DISEASE OF SANDAL (*SANTALUM ALBUM*, LINN.).

PART X. Seasonal studies on healthy and partially spiked trees.

By A. V. Varadaraja Iyengar.

It was shown in a previous communication (*J. Indian Inst. Sci.*, 1928, **11** A, 97) that the leaf tissues and tissue fluids of spiked sandal trees were abnormal in composition. In view of these findings, it was considered necessary, for a proper understanding of the disease, to study the metabolism of the leaf during the progressive stages of spike. Simultaneous observations on healthy sandal were also made and were expected to throw considerable light on such metabolic processes as vegetative growth, flowering and fruiting ; and to show by contrast the effect of the disease on spiked trees.

The leaves used in the present investigation were collected from one experimental area (Uttarahalli), and possible errors due to soil and climatic variations were thus obviated.

Four fairly large trees, two healthy and two diseased, located near each other and of nearly the same age and height, were chosen for collection of specimens. The diseased trees had a few small branches still unaffected and actually flowering. In the affected branches the internodes were shortened ; the leaves were narrower and smaller than those on the unaffected ones and no flowers were seen. In the later stages, the disease was observed to have spread to one of the healthy twigs. The healthy trees were bearing fruit in the beginning of the investigation and after June, buds developed, thereby indicating that these trees remained healthy throughout the period of investigation. To exclude differences arising from age, only mature leaves from large branches were collected for examination ; other details relating to the collection and handling of specimens are set forth in an earlier part (*loc. cit.*, 103).

The season covered by the investigation was from early April to late September in 1928 and represented a hot and dry spring, a cool and rainy summer and a mild and humid early autumn. Table I presents the weekly averages for the more important meteorological observations in the period.

TABLE I.

1928		Rainfall, inches	Sunshine, hours	Max. temp. F.	Min. temp. F.	Relative humidity per cent.
April	10	0·01	9·1	93·8	68·9	52·0
,,	20	0·06	9 0	97·0	70·5	44·0
,,	30	0·00	9·5	97·5	72·0	42·3
May	8	0·01	8·8	97·7	72·0	49·1
,,	22	0·29	9·3	96·2	71·5	49·4
June	8	0·01	6·2	87·9	68·4	61 3
,,	22	0·25	5·7	76·4	66·9	60·0
July	9	4·31	5·1	85·1	67·0	70·0
,,	23	0·40	3·1	80·9	65·9	70·0
August	8	1·73	2·8	81·7	66·3	77·3
,,	22	3·07	2 5	80·5	65·6	79·1
Sept.	7	0·02	6·6	84·6	64·8	64·1
,,	21	0·70	5·1	83·4	65·8	66·7

It may be noted that while April and May were very hot, had over nine hours of bright sunshine per day and under 50 per cent. of relative humidity, the late part of July and the whole of August were cool, had under three hours of bright sunshine per day and over 70 per cent. of relative humidity; June and September were intermediate between the two extremes. Such marked variations in season were expected to influence the soil conditions and physiological activities of the trees, and indicate the extent to which the conditions of the latter were affected in both health and disease.

Analytical Methods.

Extraction of sap was made according to the method detailed in an earlier communication (*J. Indian Inst. Sci.*, 1928, **11A**, 23) with the difference that a longer time was allowed for the material to thaw and the cells to plasmolyse, and time of extraction reduced to 10 minutes.

Freezing-point determinations were made by the micro-Beckmann method, with corrections for super-cooling. True depression of the freezing-point was calculated according to the formula $\Delta = \Delta' - 0{\cdot}0125\mu\,\Delta'$ where Δ' represented the observed freezing-point minus that of distilled water and μ the degrees of cooling below the observed freezing-point. Osmotic pressure P was calculated using the formula, $P = 12{\cdot}06\,\Delta - 0{\cdot}021\,\Delta^2$. Specific electrical conductivity, hydrogen-ion concentration and titration values were determined by methods already described (Varadaraja Iyengar, *loc. cit.*, 97).

The sap-contents were estimated by the refractometer method using Schönrock's and Geerlig's tables (*Bureau of Standards*, 1918, **44**, 133) respectively, for water and solids. The values thus obtained were checked from time to time by the evaporation method.

Extraction and clarification of the leaf-sugars were conducted by the method previously adopted. Estimations of the individual sugars were made by the method of Tollenaar Dirk (Omzettingen van Koolhydraten in het Blad van *Nicotiana Tabacum*, L., 1925, 21). Reducing sugars were estimated by the Bertrand method, 25 c.c. of centrifuged aqueous extract being used for this purpose; sucrose by hydrolysing 25 c.c. portions of extract with 1·8 c.c. of 37 per cent. hydrochloric acid at 70° for 5 minutes, neutralising, diluting to 50 c.c. and determining reducing power as in the previous case; maltose by a method similar to that adopted for sucrose, but differing in the time of hydrolysis which was extended to 24 hours.

After removing sugars with 96 per cent. alcohol, dextrins in the dried residue were extracted with 10 per cent. alcohol at 50° for 30 minutes. The alcoholic extract was concentrated to a small volume and extracted with water. The aqueous extract was clarified, filtered, treated with one-tenth its volume of hydrochloric acid (*d.* 1·125) and hydrolysed at the temperature of boiling water for 2 hours. The hydrolysate was neutralised, made up to volume and the reducing power determined as glucose. The residue from the above was used for determination of starch by the taka-diastase method.

The residue after starch determination was refluxed with 2·5 per cent. sulphuric acid for 1 hour, filtered, neutralised, clarified, made up to volume and the reducing power estimated. This was denoted as hemicelluloses calculated as dextrose.

The other chemical constituents were determined by the methods detailed before (*loc. cit.*, 98). In addition to the above, the leaves and twigs of the healthy portion of the partially spiked trees were examined histochemically for starch, and chemically for nitrogen and calcium.

The effect of season on the tissue fluids and the several chemical constituents of the tissues are presented in the following tables.

TABLE II.

Sap extraction, healthy and diseased leaves: total weight, 100 gms.

Date	Healthy			Spiked		
	Wt. of sap gms.	Wt. of residue gms.	Loss during expression of sap	Wt. of sap gms.	Wt. of residue gms.	Loss during expression of sap
April 10	59·25	35·60	5·15	57·20	39·40	3·40
,, 20	55·45	39·30	5·25	59·80	35·00	5·20
,, 30	60·65	34·00	5·35	57·50	39·15	3·35
May 8	62·35	34·65	3·00	61·55	34·90	3·55
,, 22	62·50	35·05	2·45	65·40	31·80	2·80
June 8	57·00	40·25	2·75	62·65	34·55	2·80
,, 22	49·80	46·35	3·35	55·95	40·55	3·50
July 9	57·10	40·55	2·35	...	...	...
,, 23	56·90	41·25	1·85	63·65	34·25	2·10
Aug. 8	60·45	37·55	2·00	64·45	...	...
,, 22	65·20	33·10	1·70	60·35	36·20	3·45
Sept. 7	66·20	31·30	2·50	...	...	...
,, 21	55·80	40·80	3·40	...	...	...

TABLE III.

Composition of saps (per cent.).

From refractometer readings.

Date	Healthy		Spiked	
	Water	Solids	Water	Solids
April 30	82·58	17·65	84·35	15·82
May 8	83·34	16·80	84·30	15·85
,, 22	82·35	17·84	86·13	13·95
June 8	85·09	15·10	86·79	13·35
,, 22	82·30	17·87	84·26	15·89
July 9	81·72	18·48	...	...
,, 23	82·43	17·78	87·63	12·49
Aug. 8	86·03	14·19	...	...
,, 22	84·52	15·65	87·07	13·02
Sept. 7	83·83	16·32	...	...
,, 21	81·65	18·56	...	...

TABLE III A.

Composition of Saps (per cent).

By evaporation method.

Date	Water		Solids	
	Healthy	Spiked	Healthy	Spiked
22-5-28	82·68	84·84	17·32	15·16
22-6-28	84·71	85·33	15·29	14·67
23-7-28	83·18	87·91	16·82	12·09

TABLE IV.

Freezing-point Depression (Δ), *Osmotic Pressure* (P) *and Electrical Conductivity* (K) *in Healthy and Diseased Leaf-saps.*

Date	Healthy				Spiked			
	Δ	P	$K \times 10^5$	$\frac{K \times 10^3}{\Delta}$	Δ	P	$K \times 10^5$	$\frac{K \times 10^3}{\Delta}$
April 10 ...	2·47	29·7	...	...	2·31	27·8	...	...
,, 20 ...	2·30	27·6	...	...	2·18	28·2	...	...
,, 30 ...	2·75	33·0	2625	9·55	2·49	29·9	3190	12·81
May 8 ...	2·27	27·2	2190	9·65	2·11	25·4	3295	15·60
,, 22 ...	2·43	29·2	2001	8·22	2·23	26·8	2785	12·51
June 8 ...	2·50	30·0	2521	10·08	2·20	2·64	3517	16·01
,, 22 ...	2·43	29·2	2241	9·16	2·34	28·2	2777	11·84
July 9 ...	2·28	27·4	2292	10·03	...	...	...	...
,, 23 ...	2·53	29·8	2633	10·42	2·07	20·9	2829	13·69
Aug. 8 ...	2·05	24·7	2204	10·74	...	...	...	...
,, 22 ...	2·06	24·8	2338	11·35	1·94	23·3	2439	12·59
Sept. 7 ...	2·01	24·1	2489	12·41	...	...	...	...
,, 21 ...	2·11	25·3	2426	11·52	...	...	...	...

TABLE V.

P_H and Titration Values of Leaf-saps, Healthy and Spiked.

Litmus = P_H 6·81 ; Phenolphthalein = P_H 8·37 : 4 c.c. of sap was titrated in each case against 0·966 N/10 alkali.

Date	Healthy			Spiked		
	P_H	Litmus, c.c. alkali	Phenol-phthalein, c.c. alkali	P_H	Litmus, c.c. alkali	Phenol-phthalein, c.c. alkali
April 10 ...	5·64	1·83	4·69	5·73	1·02	3·17
,, 20 ...	5·75	1·58	4·27	5·70	1·71	4·05
,, 30 ...	5·60	1·47	3·56	5·67	1·28	3·18
May 8 ...	5·65	1·90	4·50	5·85	1·12	3·03
,, 22 ...	5·75	1·57	4·00	5·83	1·33	3·40
June 8 ...	5·60	2·06	4·92	5·73	1·11	2·91
,, 22 ...	5·68	1·60	4·20	5·58	1·37	3·37
July 9 ...	5·71	1·52	3·90	...	...	...
,, 23 ...	5·54	2·18	4·64	5·51	1·23	3·02
Aug. 8 ...	5·40	2·10	4·49	...	...	...
,, 22 ...	5·35	2·17	4·41	5·19	1·80	3·83
Sept. 7 ...	5·47	1·68	3·71	...	...	...
,, 21 ...	5·40	1·83	4·21	...	...	...

TABLE VI.

Moisture-content of Healthy and Partially Spiked Leaves (per cent.).

Date	10·IV	20·IV	30·IV	8·V	22·V	8·VI	22·VI
Healthy	67·1	65·8	69·0	68·0	69·5	67·6	65·0
Diseased	70·1	69·3	69·2	69·7	75·1	72·0	69·0

Date	9·VII	23·VII	8·VIII	22·VIII	7·IX	21·IX
Healthy	66·1	67·1	65·7	69·4	67·5	65·8
Diseased	...	72·2	...	71·9	...	...

TABLE VII.

Ash, Ash-constituents and Total Nitrogen; Healthy (H) and Diseased (D) Leaves.

Percentage on dry weight.

Date		Ash		SiO_2		P_2O_5		CaO		K_2O		Total Nitrogen	
		H	*D*	*H*	*D*	*H*	*D*	*H*	*D*	H	D	H	D
April	10	12·91	16·25	0·09	4·1	0·20	0·97	3·76	0·88	1·97	2·94	2·35	4·21
,,	20	12·40	19·25	0·29	5·96	0·44	0·59	3·50	1·22	2·57	3·74	2·99	3·71
,,	30	13·61	20·98	0·56	7·11	0·21	0·74	3·53	0·99	2·97	4·08	2·74	3·67
May	8	10·72	22·96	0·32	8·49	0·23	0·55	2·96	1·04	2·29	4·11	2·82	3·63
,,	22	12·71	26·56	0·69	10·91	0·29	0·92	3·35	1·16	3·66	3·92	2·81	3·38
June	8	14·04	25·04	0·62	8·43	0·45	0·65	3·21	1·07	3·02	3·84	2·91	3·27
,,	22	13·14	21·83	0·13	7·90	0·43	0·76	2·77	1·08	2·58	3·85	2·98	3·26
July	9	12·40	...	0·33	...	0·35	...	3·37	...	2·83	...	2·53	...
,,	23	12·26	21·07	0·28	7·45	0·44	0·64	2·90	0·89	3·53	3·94	2·86	3·42
August	8	12·63	...	0·40	...	0·50	...	3·84	...	3·22	...	2·70	...
,,	22	13·12	19·31	0·51	7·73	0·41	0·76	2·84	0·99	2·94	4·57	3·03	3·77
September	7	13·61	...	0·61	...	0·49	...	3·60	...	2·88	...	2·90	...
,,	21	13·91	...	0·72	...	0·45	...	3·42	...	2·97	...	2·83	...

The diseased tissues contained larger amounts of the total ash, silica, potash and nitrogen while their lime contents were significantly lower than those of the healthy ones. The higher ash-content is to a large extent accounted for by increased uptake of silica. Chemical and histochemical examinations of the healthy-looking leaves of the partially spiked trees showed that the former were identical with those from the healthy trees.

TABLE VIII.

Sugars in Healthy and Diseased Leaf-tissues.

Percentage on dry weight.

Date	Reducing sugars other than maltose		Cane sugar		Maltose	
	H	D	H	D	H	D
April 10 ...	0·057	0·083	0·239	0·577	0·506	0·020
20 ...	0·197	0·022	0·352	0·277	0·091	0·118
30 ...	Nil	Nil	0·277	0·461	0·366	0·248
May 8 ...	,,	,,	0·062	0·120	0·284	0·086
22 ...	0·013	,,	0·052	0·061	0·078	0·068
June 8 ...	0·076	,,	0·284	0·223	0·170	0·263
22 ...	Nil	0·066	0·136	0·203	0·301	0·055
July 9 ...	,,	...	0·252	...	0·260	...
23 ...	,,	0·149	0·141	0·158	0·266	0·012
Aug. 8 ...	,,	...	0·177	...	0·342	...
22 ...	,,	Nil	0·234	0·390	0·120	0·096
Sept. 7 ...	,,	...	0·054	...	0·080	...
21 ...	,,	...	0·055	...	0·224	...

The starch and the hemicellulose contents of the affected leaves were invariably higher than those of the healthy ones. They also appeared to increase with time.

The sap extraction figures shown in Table II do not exhibit any significant difference between the healthy and diseased specimens. The total dissolved matter, either from refractometer readings or by evaporation method (Tables III and IIIa) was less in the affected

TABLE IX.

Dextrins, Starch and Hemicellulose in Healthy and Diseased Leaf-tissues.

Percentage on dry weight.

Date.	Dextrins as Dextrose		Starch		Hemicellulose as Dextrose	
	H	D	H	D	H	D
April 10 ...	1·01	2·11	0·41	0·64	5·53	6·58
,, 20 ...	...	...	0·35	0·60	4·53	6·26
,, 30 ..	1·82	2·19	0·57	0·87	5·13	6·68
May 8 ...	0·95	1·03	0.68	1·04	6·47	7·78
,, 22 ...	1·60	1·70	0·58	0·87	6·45	7·39
June 8 ...	0·33	0·86	0·14	0·69	5·52	8·08
,, 22 ...	0·15	0·22	0·25	1·60	7·98	9·49
July 9 ...	0·36	...	0·24	...	6·52	...
,, 23 ...	0·83	0·78	0·13	1·35	8·35	9·12
Aug. 8 ...	0·69	...	0·09	...	6·62	...
,, 22 ...	0·60	0·40	0·09	3·34	5·04	9·80
Sept 7 ...	0·71	...	0·05	...	5·82	...
,, 21 ...	0·68	...	0·08	..	6·25	...

specimens, indicating a diluted condition of the sap, and this is confirmed by the freezing-point determinations.

The specimens having been taken from branches at similar heights, the osmotic concentrations measured on the same dates were comparable with each other, and showed (Table IV) that the concentrations were invariably lower in the spiked than in the healthy specimens. In contrast to this, the electrical conductivity figures for the diseased specimens were higher than those for the healthy ones, pointing to a higher content chiefly of dissociated ions (electrolytes) in the former. It is possible that the higher value of the ratio K/Δ in the affected specimens indicates a greater power of absorption and retention mainly of minerals, than in the healthy ones, as also suggested by the heavier ash from the spiked leaves (Table VII).

The tissue-fluid reactions of healthy and diseased leaves (Table V) varied only slightly from each other. The titration values either to

litmus or phenolphthalein were less for the diseased tissue-fluid, indicating a lower buffering capacity than that of the healthy leaves.

The dry matter of the spiked leaf tissue was less than that of the healthy one (Table VI). The extent to which photosynthetic or respiratory activities are responsible for the diminution in dry weight in both the tissue and tissue-fluids of the spiked leaf of the partially diseased tree is a problem for further investigation. The diseased material gave also a greater ash (Table VII) which was in some samples over twice that for the healthy sample. The various ash constituents were higher in the diseased leaf with the exception of calcium which was much lower than in the healthy ones. The greater ash was largely accounted for by the increase in silica.

In the healthy leaf there was generally more maltose and less cane sugar than in the diseased (Table VIII), which contained more dextrins, starch and hemicellulose than the healthy tissues (Table IX).

There is some apparent contradiction between the present set of data and an earlier one (*J. Indian Inst. Sci.*, 1928, **11A**, 93) for the spiked specimens. In the present investigation these were drawn from partially spiked trees, and in the previous one from completely diseased saplings. In both cases the diseased samples were compared with healthy ones. The chief differences between the data of the two sets of spiked specimens were apparent in dry weight, sugars, ash and some of the ash constituents. In the case of the tissue fluids the order of the results was reversed. The only points of similarity between partially and fully spiked trees were the increase in nitrogen and starch values and diminution in calcium contents of the diseased tissues compared with those for the healthy ones.

It is interesting to note that in both cases the ratio Ca/N was much lower for the diseased leaves than for the healthy ones. Since calcium is necessary for normal leaf development, and nitrogen in large quantities induces vegetative growth, the ratio Ca/N, as observed already, should serve as an index for the diseased condition of the plant. Diminished calcium is associated with reduction in the size of the leaf and increased nitrogen with vegetative activity: deficiency in calcium and increase in nitrogen lead to a restricted root development. All these have been observed in the case of spiked sandal. According to Loew (*U. S. Dept. Agr. Bur. Plant Ind.* 1903, *Bull.*, 45) one of the first disturbances when there is deficiency of lime in a plant, is cessation of transportation of starch. Starch tends to accumulate in the lower parts of the stem so that even its transportation from the storage receptacle to the axial parts may stop ultimately. A histochemical examination of the diseased twigs of a partially spiked tree

showed a large accumulation of starch in the twigs and a small amount in the diseased leaves. In the healthy leaves and twigs collected on the same days, no such accumulation was noticed. In the healthy branches of the partially spiked tree, there was no noticed abnormality with regard to starch, nitrogen and calcium.

Except for the higher moisture-content in the samples examined during August, a month that was wet and humid with a minimum of bright sunshine, the effect of season on both healthy and diseased leaf tissues and saps seems to have been almost the same. On the whole, the chemical composition and physico-chemical properties of the sap from the healthy leaf tended to vary within a limited range from a mean value, while the corresponding data for the diseased one changed steadily with the advance of the disease. It may therefore be inferred that the metabolic changes characteristic of the different stages of the disease are determined largely by the progress of time from the onset of the disease and are not much affected by seasonal variations.

SUMMARY.

1. Tissue-fluid from the diseased leaves of a partially spiked tree contained less dissolved matter than that from a healthy one. The reaction of the former tended to become more acid, while that of the latter remained about the same throughout the period of the investigation. The osmotic concentration of the sap from the diseased specimen was less than that from the healthy ones. The electrical conductivity of the sap from the diseased leaf was generally greater than that from the healthy one and tended to increase with time.

2. In the diseased tissues greater moisture, ash and nitrogen, with less calcium than in the healthy ones, were noticed. The ratio Ca/N was markedly lower for affected than for healthy tissue.

3. Season appeared to have had a similar effect on the chemical composition of leaf tissue and on the chemical composition and physico-chemical properties of leaf-sap in health or disease.

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